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Electrospray Ionization Mass Spectrometry Analysis of Covalent and Non-covalent DNA Complexes

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Electrospray Ionization Mass Spectrometry Analysis of Covalent and Non-covalent DNA Complexes

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The covalent and non-covalent interactions between DNA and external ligands and between DNA and itself are critical for cellular function. An increased knowledge of these interactions can be used for the development of disease-fighting agents, specifically anti-cancer drugs with improved sensitivity and specificity for tumor cells. Electrospray ionization mass spectrometry (ESI-MS) is useful in the screening and characterization of the interactions involving nucleic acids given the speed and small sample sizes that can be analyzed. In this dissertation, ESI-MS is used to characterize covalent and non-covalent interactions involving DNA to assist in determining how these interactions can lead to better therapeutics.

The non-covalent binding of ligands to quadruplex oligonucleotides is discussed first. Pyrrole inosine ligands, which bind to guanine bases, were found to interact with both quadruplexes and with guanine rich oligonucleotides without a quadruplex structure.

While those interactions were specific with guanine, novel platinum complexes were found to form specific interactions with quadruplex structures themselves as the size of the ligands matched the size of a guanine quartet. This allowed the ligands to end-stack with quadruplexes with large thymine-rich loops between guanine-rich regions.

The non-covalent and covalent interactions between ligands and other DNA structures were also studied. The non-covalent binding of anthracycline ligands to mismatched DNA hairpins was probed. The analysis of solutions of approximately equimolar ligand and oligonucleotide indicated preferential binding to the mismatched sequences. Diazirdinyl benzoquinone crosslinkers, including the clinically studied RH1 and an analogue of RH1, were reacted with a variety of duplex oligonucleotides. The complexes were observed by LC-MS and dissociated using both CID and IRMPD to determine the sites of crosslinking. It was determined that both ligands could form interstrand crosslinks in DNA with 5'-GNC or 5'-GNNC sequences. The RH1 analogue, with a bulky phenyl group, formed fewer crosslinks than RH1.

In addition to studying DNA/ligand interactions, the interactions between oligonucleotides were also probed. Oligonucleotides containing non-standard isoguanine repeats were annealed in the presence of various cations to determine how those cations would affect the resulting secondary structures. In most cases, isoguanine containing strands formed pentaplexes rather than quadruplexes, which were observed for strands containing guanine bases.

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Chapter 1: Introduction

A major focus of anti-cancer research is on developing new ligands that interact with DNA. As DNA is critical in both replication and in normal cellular function, altering DNA through non-covalent or covalent binding can significantly change the cell life cycle and can result in cell death. The design of new anti-cancer ligands often involves the creation and comparison of many different variants of a given molecule followed by intensive testing to determine the best combination of efficacy in tumor-tissue and low-toxicity in normal tissue. Mass spectrometry can be a useful tool in this process. While traditional ionization methods were not able to ionize large biomolecular complexes, electrospray ionization (ESI) can transfer large, non-covalent biomolecular structures directly from the solution phase to the gas phase.[1-7] Mass spectrometry is especially useful for determining site specific binding of DNA/ligand interactions[8, 9] as well as comparing the relative affinity of different ligands towards DNA.[10-12]

This dissertation focuses on the use of ESI-MS to study covalent and non-covalent DNA complexes. In this introduction, nucleic acid structures and complexes are reviewed as well as the use of ESI-MS to study these complexes. The chapter closes with an overview of the following chapters.

1.1 DNA Structures

Nucleic acids are the basis of cellular growth and reproduction, and probing the interactions involving DNA structures is crucial to understanding cellular processes. DNA exists in several forms throughout the lifetime of the cell in response to the cellular

environment. In what might be termed a resting state, DNA is coiled in a double helix which is folded around itself in a supercoil. Supercoiled DNA wraps around histones, which allows the cell to collapse a massive biomolecule into an efficiently packed structure.[13] During replication or transcription, the supercoil is unfolded and the helix is unzipped to expose the bases at the center of the helix.

While the overall structure of cellular DNA is a double stranded helix, there are many variations of and changes that occur to this core structure that are important to cellular function. The helix itself can take several forms depending on the environmental conditions as well as the DNA sequence. For example, A and B form DNA are both right handed helices, but the A form, which occurs under non-physiological conditions such as when hydration is low, is wider than B DNA and has a shallower minor groove (Figure 1.1).[14-16]

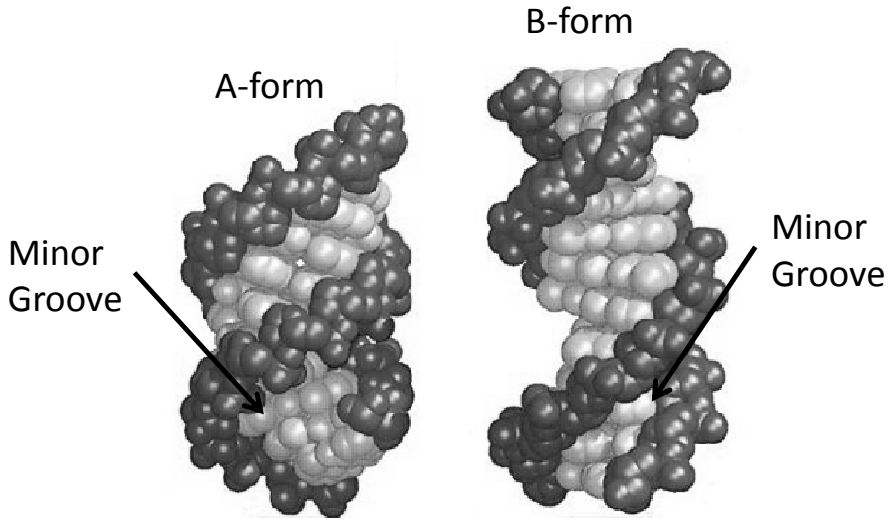

Figure 1.1 Cartoon approximations of A and B form DNA. Both are right handed helices. Adapted from http://www.biochem.arizona.edu/classes/bioc461/Chapter27Notes.htm#A-DNA

In addition to the common duplex, some segments of DNA can be single stranded. This most notably occurs during transcription or replication when the DNA helix opens to allow for the binding of DNA replication enzymes. Another structure found in the cell is the triple-stranded helix. Triple stranded DNA involves a short oligonucleotide binding in the major groove of B form DNA. The B-form DNA is stabilized by canonical base pairs. The third strand is bound to the helix through Hoogsteen or reverse Hoogsteen hydrogen bonding.[17, 18] Hoogsteen interactions are alternatives to Watson-Crick base pairing and involve interactions between bases through different functional groups (Figure 1.2). Hoogsteen interactions can occur between traditional Watson-Crick base pairs such as adenine and thymine as well as nontraditional base pairs such as guanine bound to guanine or guanine bound to adenine. For example, a repeat of $d(GA)_n$ in a B-form duplex with the complementary $d(CT)_n$ strand could have another $d(CT)_n$ strand bound in the minor groove of the duplex.[17, 19] It has been shown that triplexes are important in the regulation of transcription and replication.[18]

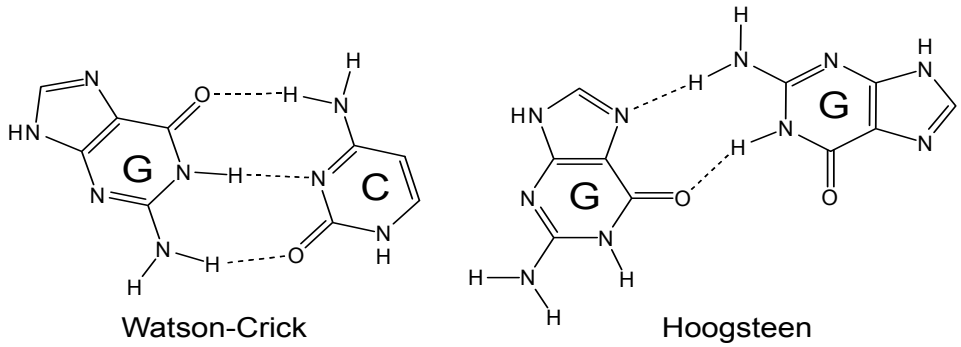

Figure 1.2 Two types of base pairing involving guanine bases. Hoogsteen binding between guanines is the basis for G-quartet formation.

Other structures adopted by physiological DNA are modulated by sequence. For example, cytosine rich DNA can form i-motif structures under low pH conditions. In this structure, protonated and neutral cytosine bases interact to form a non-canonical base pair and a non-helical folded structure.[20-22] However, the most well known non-duplex structure is the guanine quadruplex. Quadruplexes are formed from guanine rich DNA strands that often consist of guanine repeats separated by thymine and adenine rich regions. The basis of a G-quadruplex is formed by the interaction of four guanine bases. Rather than forming conventional Watson-Crick base pairs with cytosine, the four guanines interact with one another through Hoogsteen interactions, and form a planar quartet.[23-26] In most cases, the guanine bases surround a central cation that, physiologically, is often potassium or sodium (Figure 1.3a).[27-29] There is a large array of possible quadruplex structures based on the sequences of the strands involved as well as the environmental conditions. Specifically, quadruplexes can be inter- or intramolecular and multiple conformations of intramolecular quadruplexes are possible (Figure 1.3b).[23, 25, 26, 30-35] G-quadruplexes have been intensively studied due to their role in replication. Telomeres, non-coding guanine rich regions at the ends of DNA, allow the cell to replicate multiple times and shorten each time the cell replicates. In immortal cancer cells, telomeres maintain their length despite repeated replication cycles which permits many more replications to occur before cell death is initiated.[36] Telomerase, the protein that lengthens telomeres and thereby extends the life of a cell, can only bind to the single

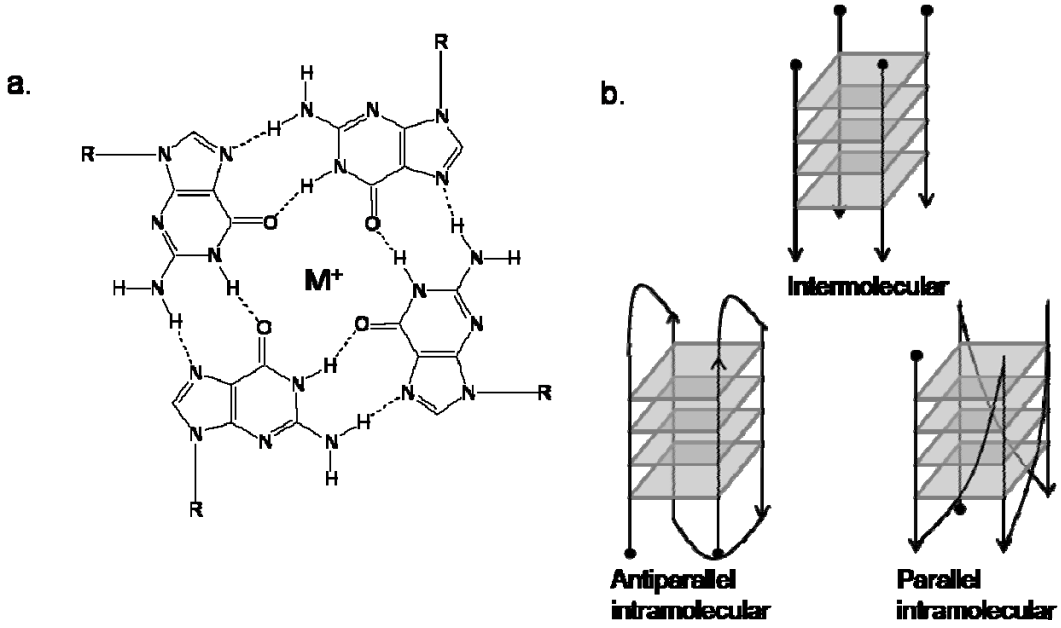

Figure 1.3 a. Structure of a guanine tetrad around a central metal cation. b. Schematic of intermolecular and intramolecular quadruplexes. Arrow heads point to the 3' end of the nucleic acid strands.

strand conformation of the telomere.[37] If the quadruplex conformation of the telomere is stabilized, telomerase cannot bind and the lifespan of the cell can be limited- an important factor in controlling cancer.[36, 38-40]

While biological DNA structures have obvious import, non-biological structures are also of interest. The ability to self-assemble and the variety of structures that nucleic acids and DNA can take make DNA an option for sensing and ion transport applications.[41-47] DNA microarrays in which immobilized arrays of single strands are hybridized with fluorescently labeled target DNA in solution. After washing, the fluorescent areas of the array can be excised and sequenced. This technique is used in genomics for gene expression and discovery.[46] The structure of DNA itself can also be used in ion transport applications. For example, the ability of guanine rich nucleic acids

to self-assemble into guanine quartets has been applied to the formation of passive transport channels.[44, 47] Once modified nucleotides are allowed to form into quartet structures, the central cavity permits small cations to traverse the channel while larger compounds cannot. Using bases other than guanine can change the dynamics between the bases and create ion channels of other sizes.[42, 45, 48]

1.2 DNA STRUCTURE BY ESI-MS

While mass spectrometry cannot directly image the structures formed by DNA strands, a great deal of information can be gained through interrogation by mass spectrometry. The most basic information is obviously the size of the complex and the number of strands involved which is readily determined based on the mass-to-charge ratio of the ions observed in the mass spectra. However, mass spectrometry has also been used to determine the relative stabilities of gas-phase quadruplexes.[49] Also, ESI-MS has been used to characterize the number of cations bound in the central cavities of quadruplexes.[50]

Also of great use in the study of DNA structures has been ion mobility mass spectrometry.[51-55] This technique involves the ionization of the molecules under study followed by allowing the ions to travel through a gas-filled chamber before mass-selective detection. In addition to gaining information on the mass to charge ratios of the analytes, the analytes separate by the size, shape and charge of the ions while traveling in the drift tube. With this technique, it was determined that gas phase A-T base pair rich duplex DNA is closer to B-DNA while gas phase G-C rich DNA is more like A-form

DNA.[54, 55] In addition, the smallest duplex that can retain a duplex conformation into the gas phase, a minimum of eight base pairs are required, was determined as well.[54, 55] Quadruplex structures based on the human telomeric sequence were studied by ion mobility mass spectrometry and were shown to remain in a quadruplex structure in the gas phase.[52, 56]

1.3 DNA/LIGAND COMPLEXES

DNA interactive agents have been studied for their use as anti-cancer and anti-bacterial drugs.[40, 57-60] While DNA binding varies from non-specific interactions to interactions based on sequence and local structure, most therapeutic DNA binding is specific. Since alteration of DNA though ligand binding changes the function of the cell, the ideal drugs would selectively bind only to DNA in diseased cells to limit any effects in normal tissues. This need for selectivity is especially great in cancer cells since they are essentially normal cells that have begun replicating aggressively.

Non-covalent DNA binding ligands can bind in several ways (Figure 1.4). Electrostatic binding ligands bind non-specifically to the outside of DNA duplexes. The negative charge of the sugar-phosphate backbone can allow for positively charged ligands to associate with the duplex without base interactions. Molecules that exhibit this kind of binding include metal cations, such as sodium or magnesium, as well as small cationic ligands such as 1,3-propane diammonium. In most cases, multiply charged cations bind more strongly than singly charged cations.[61] Minor groove binders do form specific interactions with the narrow shape of the minor groove at A/T rich sites. These

Figure 1.4 Illustrations of common non-covalent DNA interactions.

types of ligands, such as distamycin and netropsin, form hydrogen bonds with bases in the minor groove.[62-65] The groove at G/C rich regions is wider and the interactions with ligands aren't as strong. Another type of binding is intercalation. DNA intercalators are generally planar ligands that are capable of fitting between stacked bases within the duplex.[58, 60, 66] Two molecules that bind intercalatively are ethidium bromide and daunomycin. The intercalating ligands are bound by π stacking interactions between the ligand and bases. With duplexes, intercalation causes distortion of the helix and duplex lengthening as the bases must adjust to fit the ligand between stacked bases.[16, 61] Intercalation is most commonly found in G/C rich regions and can also be seen with G-quadruplexes. However, ligands that interact with quadruplexes most often do so by base stacking to the top or bottom of the quadruplex rather than intercalation as the energetic cost of insertion into and disruption of the quadruplex is too great.[67, 68]

There are many ligands that form covalent attachments to DNA, primarily at reactive sites on the nucleobases. Binding can occur at all four nucleobases and at different sites on those nucleobases, but several sites are more nucleophilic and more

reactive. The most nucleophilic site on any of the bases is the N7 position on guanine. This is a frequent site of covalent adduction by small molecules such as dimethyl sulfate (Figure 1.5a) as well as larger molecules such as nitrogen mustards.[69] Another reactive site is the N3 of adenine which sits in the minor groove. The antibiotic duocarmycin and its analogues bind at this site (Figure 1.5b).[70, 71] Anti-cancer and antibiotic agents are often based on a covalent binding functionality since covalently bound adducts can lead to errors in the replication process that induce apoptosis in cancer cells. Covalent binding can refer to simple methylation of a base[72] or to covalent crosslinking between the two DNA strands[73, 74] or between a DNA strand and protein.[75-77] DNA interstrand crosslinks are particularly damaging to the cell as they prevent unzipping of the duplex which results in a failure to transcribe or replicate. Crosslinking agents have been used clinically, and many new crosslinking anti-cancer ligands are under investigation.

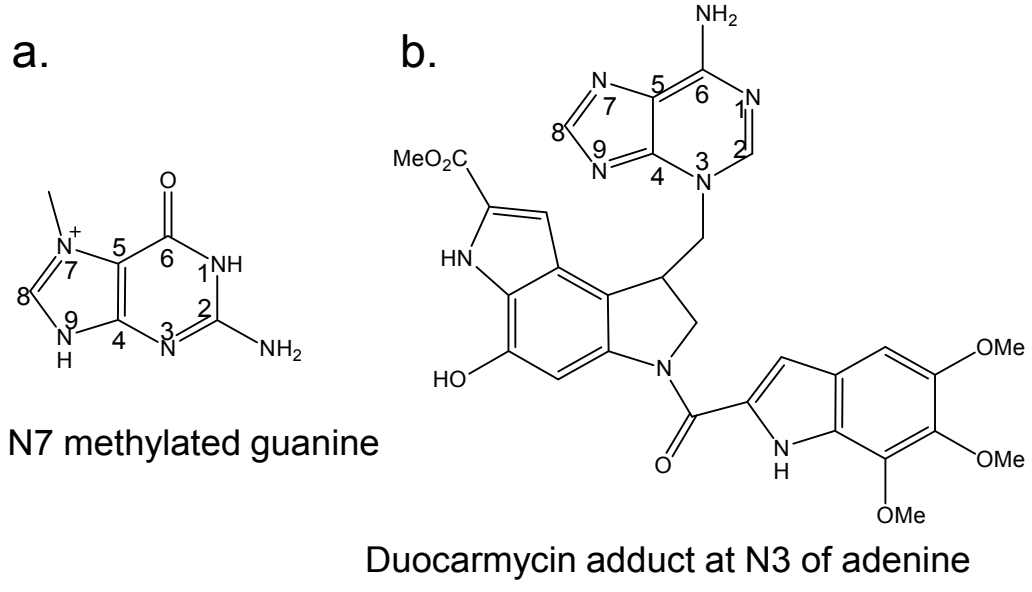

Figure 1.5 Examples of covalent modifications to guanine (a) and adenine (b).

1.4 ESI-MS OF DNA/LIGAND COMPLEXES

ESI-MS is a versatile method for analyzing DNA/ligand complexes. Many aspects of binding can be assessed from a mass spectrum, and the technique is fast compared to gel electrophoresis allowing for high-throughput analysis. The relative binding affinities of various ligands can be compared based on a comparison of the ion abundances for different DNA/ligand complexes,[10, 12, 78-80] and the stoichiometries of the DNA/ligand complexes are readily apparent based on the m/z values of the complexes. The results are acquired in a non-native gas-phase environment, but ESI affords a fast, gentle means of transferring the complexes into the gas-phase with minimal disruption of non-covalent interactions.

While the determination of selectivity and stoichiometries of non-covalent complexes is readily performed, determining the site of reactivity can be more difficult. Reactions at different sites have the same mass so tandem mass spectrometry must be used to generate fragmentation patterns to probe the structures of the complexes. However, the most common dissociation event for non-covalent DNA/ligand complexes is often ejection of the ligand, which does not indicate the site of binding. To provide information about the location of non-covalent binding, chemical probes techniques can be used. Chemical probes are small molecules that covalently attach to a target site and are used to study structures and interactions in nucleic acid and protein complexes. Chemical probes and footprinting reactions have often been used to probe ligand binding sites and DNA conformations in solution.[81-85] In footprinting reactions, probe molecules are added to solutions containing the DNA/ligand complexes and the probes are allowed

to covalently bind. The DNA is then digested and sequenced to determine where the probe was able or unable to react. These unreacted sites indicate where the ligand was bound along the DNA.[82, 84, 85] While informative, these reactions are usually analyzed by gel electrophoresis which can have lower resolution and take longer than mass spectrometric analysis. For a mass spectrometric alternative, covalent binding between the chemical probe and the DNA/ligand complex is performed in solution as usual. Following clean up, the solution is analyzed by mass spectrometry to detect complexes containing the chemical probe. The DNA/chemical probe complex is then dissociated to pinpoint the site of reaction based on the fragmentation pattern and the observed mass shifts in some of the product ions. In the case of duplex/ligand binding, the ligand is allowed to incubate with the DNA for a period of time before addition of the chemical probe. As before, the sample is cleaned up and analyzed to detect the DNA/ligand/probe complexes and dissociation is performed on those complexes. For these complexes, the presence of greater reaction in a given region can signify local unwinding of the helix which indicates the non-covalent binding site.[86]

Covalent DNA/ligand interactions have also been studied with mass spectrometry. One technique used to characterize covalent modifications is the digestion of DNA to create individual bases. LC-MS/MS of the resulting bases can provide information on the types of bases that are covalently modified, based on the change in mass, as well as detailed structural information about the modification but cannot provide information as to where the modification occurred within the sequence.[87-91] Crosslinked DNA can also be analyzed in the same fashion.[87, 92-94] Alternatively, intact duplex/ligand adducts can be

directly subjected to tandem mass spectrometry, and the resulting fragmentation patterns can be interpreted.

1.5 DISSOCIATION TECHNIQUES FOR DNA

An advantage of mass spectrometry is the ability to gain structural information for a given analyte by dissociating the ion into fragments. The most common method of dissociation in mass spectrometry is collisionally induced dissociation (CID). To perform CID in an ion trap, the precursor ion is isolated within the trap. Application of an AC voltage causes translational excitation of the ion, leading to energetic collisions with the helium buffer gas. The collisions cause conversion of kinetic energy to internal energy promoting dissociation in a distinct fragmentation pattern for that ion. CID is a low energy fragmentation technique which, for single stranded DNA, often results in loss of a base-a relatively uninformative fragmentation pathway. Upon CID, duplexes can dissociate through two pathways: by base loss similar to single strand DNA or by strand separation to the individual strands.

Infrared multiphoton photodissociation (IRMPD) is another means to dissociate ions. With IRMPD, photons, usually from a CO_2 laser at a wavelength of 10.6 μm, are used to activate the precursor ion. This requires that the precursor ion be capable of absorbing photons at the wavelength of the laser. Phosphate groups and sulfate groups both absorb at 10.6 μm making DNA, with its sugar-phosphate backbone, highly chromogenic at this wavelength. Both single stranded and double stranded oligonucleotides have been dissociated by IRMPD,[95] and complexes between duplexes

and small molecules have also been studied.[96, 97] There are advantages to IRMPD in comparison to CID that apply to all molecules and that apply to DNA in particular. CID requires collisions with helium molecules and excitation of ion trajectories which can scatter the ions and lower signal. In addition, in order to maximize the efficiency and energetics of the collisions, the trapping voltages are raised to a level that prevents trapping of lower m/z ions. This can result in the loss of informative low mass fragments. In comparison, in IRMPD the lack of energetic collisions and the absence of excitation of the kinetic energies of the ions prevent loss of ions due to scattering. Also, the trapping voltages are not raised during IRMPD thus retaining low mass ions. Since the fragment ions are also irradiated during activation, IRMPD can result in multi-step fragmentation, termed secondary fragmentation, which gives more information about the structure of the precursor ion. Since the activation waveform for CID is only applied to the precursor ion, multistage fragmentation requires step-by-step isolation and dissociation of individual fragment ions which is often limited by signal intensity. For DNA specifically, the secondary fragmentation mechanism of IRMPD gives much more sequence information than CID, the latter which is dominated by base loss. As with CID, the primary fragmentation pathway in IRMPD is often base loss. However, these base loss ions readily absorb IR photons and are converted to informative sequence ions, making IRMPD a powerful tool for DNA analysis.

Recently, other dissociation methods have been successfully used for the characterization of DNA. While not further discussed in this dissertation, it is useful to briefly highlight some of their advantages. Electron based dissociation methods have

become popular in mass spectrometry in recent years.[98-101] Electron capture dissociation (ECD),[102, 103] which uses an electron beam to energize and dissociate ions via an exothermic electron transfer process, and electron transfer dissociation (ETD),[104-107] in which reagent anions are allowed to react with analyte cations to cause an exothermic electron transfer process, have become popular dissociation methods especially in proteomics applications. However, since these methods involve cationic analytes, they are not often used with DNA. Recently, DNA cations have been interrogated by ECD[103, 108, 109] and ETD.[110] Combining the electron-based activation process with collision induced dissociation provided a wide range of backbone fragments not usually observed with CID alone. In the negative mode, electron detachment dissociation (EDD) using a UV laser also provides novel backbone fragments due to the creation of radical intermediates.[111, 112]

1.6 Overview of Chapters

This dissertation is intended to further exploration of the uses of ESI-MS for the analysis of oligonucleotide complexes. Chapter 2 is a brief description of some of the techniques used in the experiments performed for this work. Both Chapters 3 and 4 discuss the non-covalent binding of novel ligands to quadruplex DNA. In Chapter 3, a pyrrole inosine ligand, which can form Hoogsteen interactions with guanine, was found to bind selectively to G-quadruplexes and to guanine rich oligonucleotides. Two control ligands either with no Hoogsteen binding functionality or with a steric block to binding showed similar levels of binding to all oligonucleotides.

Chapter 4 focuses on transition metal complexes as non-covalent G-quadruplex binding ligands. Due to the extensive π bonding surface area of the Pt ligands, the ligands showed increased binding to guanine quadruplexes over smaller Ru based complexes. The length of the thymine loops between guanine repeats had a significant effect on ligand binding. Little binding was observed with single strand or duplex oligonucleotides.

In Chapter 5, the use of two non-covalent ligands to bind preferentially to thymine mismatches was studied. The ligands, doxorubicin and daunorubicin, were studied with hairpin oligonucleotides that either consisted of a wild-type sequence or sequences with one or two cytosine to thymine mutations. The ligands exhibited greater affinity for the mismatched hairpins over the wild-type hairpins at equimolar concentrations.

Chapter 6 focuses on the covalent crosslinking of duplex DNA through benzoquinone-based crosslinkers. The two crosslinkers under study can for interstrand crosslinks at the N7 site on guanine bases. Crosslinking was determined to be sequence dependent with a minimum of one base pair required between opposite strand guanines required for crosslinks to form. A large phenyl group on one of the crosslinkers minimized the ability of that ligand to form crosslinks. Both the local and global secondary structures of the duplexes were determined to be important for crosslink formation.

While the other chapters concern ligand binding, Chapter 7 focuses on the structures of oligonucleotides themselves. Specifically, strands containing a non-standard base, isoguanine, were studied to determine the effects of annealing the strands

in the presence of different cations. While it is known that isoguanine bases prefer pentad structures over the tetrad structures favored by guanine bases, it was unclear how changing the annealing cation would affect the final oligonucleotide structures. In most cases, singly and doubly charged cations resulted in the formation of pentaplex structures. However, potassium and, under certain conditions, ammonium, could facilitate the formation of quadruplex structures.

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Chapter 2: Experimental Methods

All mass spectrometry described in the following chapters was performed with electrospray ionization (ESI) which allows for gentle ionization of large, non-covalent biomolecular complexes. This chapter describes DNA annealing conditions and briefly outlines the instrumental conditions for the mass spectrometric and solution phase experiments. Fragmentation nomenclature for nucleic acids is also described.

2.1 DNA Annealing Conditions

In order to form the non-covalent interactions between oligonucleotides, samples were annealed in the presence of salts. The cations present in the salts facilitate strand interaction by limiting the repulsive effects of the negatively charged phosphate backbone. In most cases, the salt used was ammonium acetate. While sodium or potassium buffers are usually used in order to best replicate cellular conditions, ammonium acetate is a volatile salt. The ammonium readily separates from the DNA backbone during ESI and leaves the DNA ions mostly adduct-free for mass analysis while sodium or potassium would remain bound to the DNA and lead to a complex distribution of DNA ions containing one or more metal ions. Standard oligonucleotide strands were synthesized on a 1 μmole scale and desalted by IDT DNA, Inc. (Coralville, IA). The strands were resuspended in water and the concentrations confirmed by UV/Vis spectroscopy at 260 nm. To form duplexes, complementary oligonucleotide strands at 500 μM were combined to form duplexes in the presence of 150 mM ammonium acetate. The samples were heated to 90-95°C briefly and allowed to cool to room temperature in a

water bath overnight. The annealing procedure for quadruplexes and other higher order structures was similar although both the strand concentrations and salts were often adjusted.

2.2 MASS SPECTROMETRY

All mass spectrometric experiments described in this dissertation were undertaken on ion trap mass spectrometers. Traditional 3-D ion traps have the advantages of ruggedness, low cost, and the ability to undertake multiple stages of MS in the same trapping region. After the ionization source and ion focusing optics that are common to all mass spectrometers, ions enter the ion trapping region through an entrance lens. The trap itself consists of a hyperbolic ring electrode to which an RF voltage is applied. On either side of the ring electrode are entrance and exit endcaps which are typically grounded. If the RF frequency is ramped, the ions become excited in a mass-to-charge dependent fashion. The excited ions fall out of the central ion cloud and eventually are ejected from the trap to be detected. A recent incarnation of the ion trap is the 2-D or linear ion trap. In this case, the trap consists of a quadrupole mass analyzer. RF voltages are applied to opposite rods to create a field that keeps the ion motion along the axis of the quadrupoles. To trap the ions, voltage offsets at either end of the quadrupoles are applied. These voltages can be raised, to trap the ions, or lowered, to permit ions to enter or exit the trap. The ions are ejected radially through slits in the quadrupoles and two detectors are used to improve sensitivity. In addition, the trap geometry itself improves sensitivity over the 3-D trap as more ions can occupy the space without interacting with

one another. Additional improvements to scanning speed have also been noted, and variations in how voltages are applied to the ion trap have allowed for exciting changes in trapping capabilities. For example, two ion populations with different polarities can be trapped simultaneously in the same trap.

In this dissertation, experiments were undertaken on Thermo Scientific (San Jose, CA) LCQ Duo or LTQ XL mass spectrometers. Most work was done in negative ionization mode with low heated capillary temperatures, around 90°C, to allow for desolvation of the ions while retaining non-covalent complexes. Analyte concentrations were around 10 mM in 50 mM ammonium acetate. The ammonium cations replace sodium adducts that electrostatically bind to the backbone of DNA and then are evaporated off during the desolvation/ionization process.

2.3 LC-MS

The addition of liquid chromatography is a logical extension of ESI-MS. While ESI excels at the analysis of large biomolecules, excessive salts and complex matrices are problematic. With LC providing sample clean-up as well as separation of the mixture components, the eluent can be directly analyzed by ESI-MS. For good separation of oligonucleotides, an ion pairing reagent is often added to the mobile phase in LC separations. To stay compatible with ESI-MS, 10mM ammonium acetate was used as the aqueous component to the mobile phase. The organic component was 50% acetonitrile/50% 10 mM ammonium acetate. The use of aqueous ammonium acetate in the organic phase was due to the relatively high, relatively similar polarities of the

oligonucleotides and complexes. It permitted a slow, controlled gradient for elution. A Dionex (Sunnyvale, CA) Ultimate 3000 capillary HPLC system with an Agilent (Santa Clara, CA) Zorbax 300SB C18 column (150mm x 0.3 mm, 5 μm particle size) was used for all experiments. The UV trace was collected at 260 nm.

2.4 NUCLEIC ACIDS DISSOCIATION

In the mass spectrometric analysis of nucleic acids, fragmentation is often used to further characterize the nature of the interactions. Terminology has been developed to label the fragments resulting from dissociation. In low energy fragmentation regimes, such as collisionally induced dissociation (CID) and infrared multiphoton photodissociation (IRMPD), the most common initial fragmentation from a single stranded oligonucleotide is often base loss in which the bond between the base and the sugar is cleaved. After base loss, the fragment can be dissociated further at the site of the base loss resulting in cleavage of the phosphate backbone. This fragmentation normally occurs 3' to the sugar at the carbon-oxygen bond resulting in fragments containing the 5' end with a loss of the base, a-base ions, and fragments containing the 3' end, w ions (Figure 2.1). The fragment ions are further categorized by the number nucleotides contained within the fragment which is described by a subscript. In higher energy fragmentation spectra, other ions are present including c and y ions, which are formed after dissociation 3' to the phosphate, and a ions that retain the nucleobase.

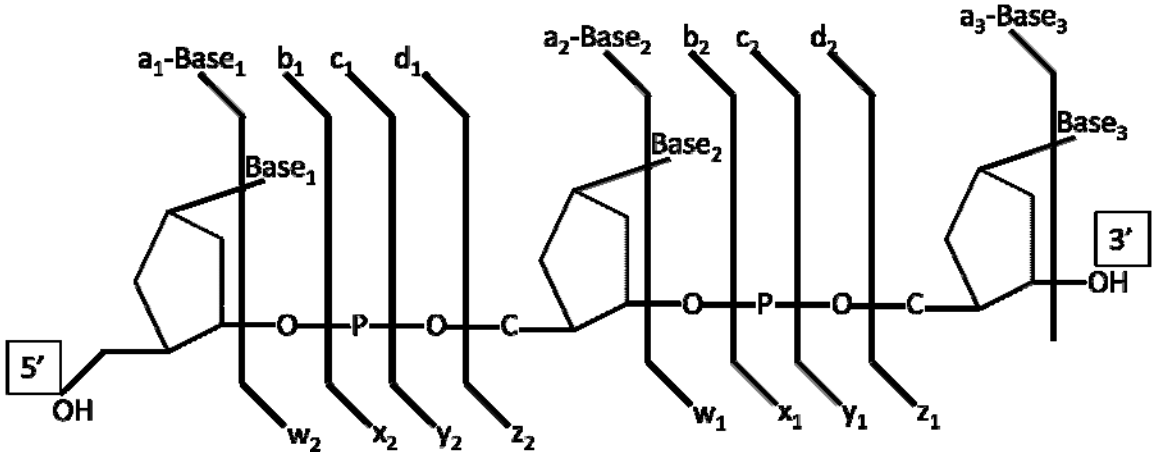


Figure 2.1 Fragmentation nomenclature for DNA dissociation.

2.5 IRMPD

IRMPD uses low energy photons from CO_2 lasers to energize ions and cause dissociation via low energy pathways. IRMPD has been shown to be an excellent tool for the dissociation of oligonucleotides. In addition to sulfate groups, phosphate groups absorb the energy of a CO_2 laser well. As nucleic acids contain a sugar-phosphate backbone, there are multiple chromophores for possible absorption of a photon. IRMPD was performed using a 50 W Synrad (Mukilteo, WA) CO_2 laser. The emitted wavelength was 10.6 μm. For the DNA experiments, the laser was operated at 20% power or 10 W, nominally. The irradiation time was 1-10 ms, and the laser was triggered during the activation step of the mass spectrometer.

2.6 CIRCULAR DICHROISM

Circular dichroism is a form of spectroscopy in which circularly polarized light is used to interrogate a sample. The difference in absorption of left and right circularly polarized light provides information on the relative orderedness of a given sample. Molecules that are chiral or have some inherent ordered structure, such as the stacking of base pairs in a duplex oligonucleotide, will result in a difference in absorption at certain wavelengths. When taken together, the resulting spectrum can give information about the overall structure of a molecule or complex. While minor details about the structure are often not represented, the technique provides a valuable way to gauge the formation of complex. Circular dichroism experiments were performed on a Jasco, Inc. (Easton, MD) J-815 spectropolarimeter. Spectra were collected over a wavelength range of 220 to 320 nm, and 3 to 5 spectra were averaged to create the final spectra. The quartz cuvette used had a 1 mm pathlength and solutions were generally 10 μM in concentration. Blank spectra of the buffers were subtracted.

Chapter 3: Characterization of the Binding of Pyrrole Inosine Ligands to Quadruplex Oligonucleotides

3.1 Overview

Based on binding studies undertaken by electrospray ionization-mass spectrometry, a synthetic pyrrole-inosine nucleoside, **1**, capable of forming an extended three-point Hoogsteen-type hydrogen-bonding interaction with guanine, is shown to form specific complexes with two different quadruplex DNA structures $[dTG_4T]_4$ and $d(T_2G_4)_4$ as well as guanine rich duplex DNA. The binding interactions of two other analogs were evaluated in order to unravel the structural features that contribute to specific DNA recognition. The importance of the Hoogsteen interactions was confirmed through the absence of specific binding when the pyrrole NH hydrogen-bonding site was blocked or removed. While **2**, with a large blocking group**,** was not found to interact with virtually any form of DNA, **3**, with the pyrrole functionality missing, was found to interact non-specifically with several types of DNA. The specific binding of **1** to guanine rich DNA emphasizes the necessity of careful ligand design for specific sequence recognition.

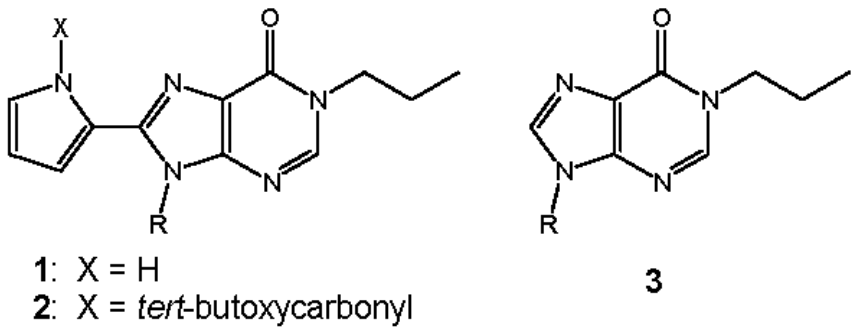
3.2 Introduction

A variety of DNA interactive ligands have been developed with a wide range of binding modes and targeted applications[1-4] such as ones designed to stall replication or transcription[2, 5, 6] or to stimulate apoptosis[7]. Common binding modes of these ligands to duplex DNA include minor groove binding and intercalation[2-4, 8]. The former is common in the deep well of adenine/thymine rich regions, whereas the latter most often occurs in

guanine/cytosine rich regions and involves insertion between base pairs in duplex DNA. Other ligands have been developed to bind to quadruplex DNA to limit binding and activity of telomerase[9-18]. There continues to be considerable effort to design and construct new DNA interactive agents, ones with greater selectivity for certain DNA structures or ones with higher affinities. The development of these DNA interactive ligands is facilitated by rapid, sensitive analytical screening methods that provide diagnostic feedback about key issues like binding selectivity and specificity of DNA recognition. Electrospray ionization mass spectrometry (ESI-MS) is one of the newer tools used to evaluate DNA interactive ligands and offers the advantages of high sensitivity, low sample consumption, and high throughput screening[19-21]. Mass spectrometric studies of DNA interactive ligands have been effective in characterizing binding selectivity and specificity[9-11, 16, 22], establishing the mode of binding[10, 11, 16, 22], and correlating specific binding patterns with *in vitro* antitumor and antibacterial cytotoxicity[22].

Recently, work presented by our group[10, 23] and others[11, 16-18, 24-27] has established the utility of using ESI-MS to evaluate the binding properties of potential quadruplex-interactive ligands. Although many of these prior studies have focused on DNA interactive ligands that have been developed as drugs, ESI-MS can also play a role in evaluating new DNA binding modalities in the early stages of ligand design, whether for new drug platforms, DNA sensors, or targeted DNA cleavage agents. Because ESI-MS offers fast analysis with minimal sample usage, it can be used conveniently to provide

step-by-step feedback at each state of ligand design, thus allowing systematic development of structure/binding activity relationships and optimization of DNA recognition.

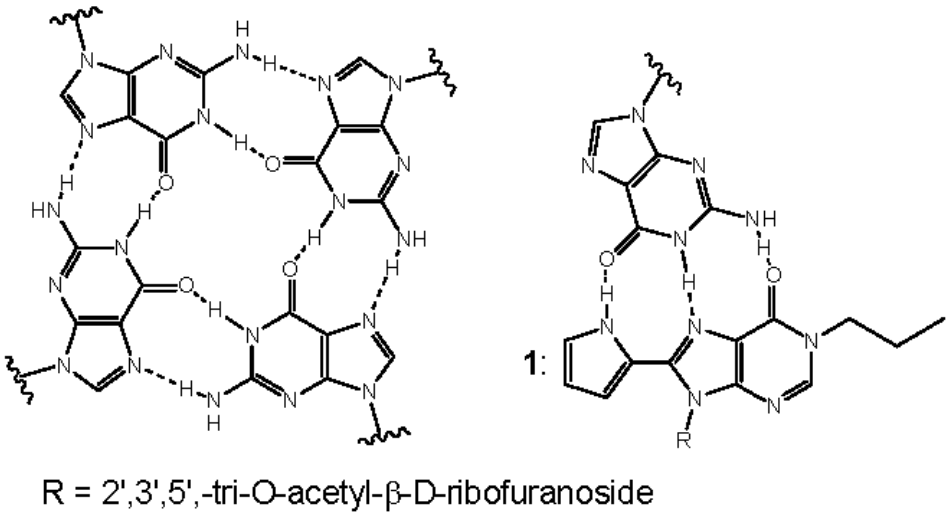


1: X = H
2: X = *tert*-butoxycarbonyl

3

Scheme 3.1 Structures of synthetic nucleosides, 1-3.

In this study, we use ESI-MS to examine a pyrrole-inosine based synthetic nucleoside, **1** (Scheme 3.1) that is an offspring from a previous water insoluble analog that demonstrated the ability to bind DNA via an extended Hoogsteen-type interaction[28]. **1** was designed with appropriate functional elements to engage in a series of hydrogen-bonding interactions with a complementary nucleobase, in this case guanine (Scheme 3.2). The present work was undertaken to determine whether **1** binds quadruplexes specifically due to their guanine-rich composition and Hoogsteen-stabilized conformations or has a more general affinity for guanine bases. The interactions of **1** with two different quadruplex-forming oligonucleotides, dTG_4T, which forms a four-



Scheme 3.2 Hoogsteen binding in guanine tetrads and between **1** and guanine bases.

stranded intermolecular quadruplex $[dTG_4T]_4$, and $d(T_2G_4)_4$, which forms an intramolecular quadruplex, as well as several single-stranded and double-stranded oligonucleotide sequences were investigated to probe the selectivity of **1** towards different DNA secondary structures. Single strands and duplexes were chosen with both high and low GC content to determine guanine selectivity. Furthermore, comparative experiments were carried out to evaluate the binding of structural analogs of **1** in which the pyrrole NH was either removed (**3**) or the NH was blocked (**2)** (Scheme 3.1).

3.3 EXPERIMENTAL

3.3.1 Reagents

The general preparation strategy for the synthetic nucleosides **1**, **2**, and **3**, albeit bound to different sugars, was described previously by Sessler et al.[28]. Oligonucleotides were prepared and purified by reversed-phase HPLC by Integrated DNA Technologies (Coralville, IA). Ammonium acetate (98% purity) and A.C.S. Grade Spectranalyzed methanol was obtained from ThermoFisher Scientific (Waltham, MA). All purchased chemicals were used without further purification. Water was purified in house with an EASYpure UV deionizer (Barnstead International, Dubuque, IA).

3.3.2 Preparation of Samples for Analysis.

Annealing of $d(T_2G_4)_4$ (Q1), $[dTG_4T]_4$ (Q2), dGGCGTCGGCGTCGC/dGCGACGCCGACGCC (DS1), and dATAAAAACGAAAATA/ dTATTTTCGTTTTTAT (DS2) was performed by heating a 1 mM solution of the oligonucleotide in 150 mM ammonium acetate to 90°C followed by cooling to room temperature over 2 hours[9, 17, 28]. Two single strand oligonucleotide sequences were also analyzed: dGGCGTCGGCGTCGC (SS1) and dATAAAAACGAAAATA (SS2). The annealed or single strand oligonucleotides were analyzed in a final ammonium acetate concentration of 50 mM and a solvent composition of 20% methanol. A ligand (20-50 μM) was incubated with DNA (10 μM) for 10 minutes prior to ESI-MS analysis.

3.3.3 Methods and Instrumentation.

Mass spectrometric analyses were performed on a ThermoFinnigan LCQ Duo quadrupole ion trap mass spectrometer equipped with the stock ESI source (San Jose, CA). Oligonucleotide samples were analyzed in the negative ion mode with the heated capillary set at 80°C. Source conditions were tuned to minimize in-source fragmentation of complexes with a needle voltage of 3.5 kV and a nitrogen sheath gas of 40 arbitrary units. Solutions were infused directly at 3 μL min^{-1}, and the spectra presented represent the average of 100 to 200 scans. Relative binding affinities for each ligand to each type of DNA are given as the fraction of bound DNA, expressed as a percentage, which relates the total peak areas of the various ligand:DNA complexes to all DNA species (both bound and free). The equation, which has been used in previous studies[23, 29], to calculate the fraction bound DNA is:

$$Fraction\ of\ Bound\ DNA = \frac{PA_{1:1} + PA_{1:2} + \cdots}{PA_{DNA} + PA_{1:1} + PA_{1:2} + \cdots} X\ 100$$

where $PA_{n:m}$ represents the peak areas of the various ligand:DNA complexes and PA_{DNA} denotes the total peak area of free DNA ions. Peak areas were determined in Origin 7.0 (OriginLab, Northampton, MA). A high fraction bound value indicates a higher ligand:DNA binding efficiency. Measurement of the peak areas, rather than relative abundances, of the complexes permits the inclusion of salt adducts that may not be evident upon visual inspection of the mass spectra.

3.4 RESULTS AND DISCUSSION

3.4.1 Interactions with Quadruplex DNA.

Previous experiments have demonstrated the utility of ESI-MS for analyzing quadruplex DNA structures formed from short oligonucleotides[10, 11, 16-18, 23, 30, 31]. For the present study, two of these oligonucleotides were selected to evaluate the interactions between quadruplex DNA and the three nucleosides. The first oligonucleotide, $d(T_2G_4)_4$, forms an intramolecular quadruplex in which one strand is folded into a quadruplex with four G-tetrads (Q1). The second oligonucleotide, dTG_4T, forms a parallel intermolecular quadruplex involving four strands of DNA (Q2), $[dTG_4T]_4$.

Examples of the ESI-mass spectra obtained for the quadruplex and quadruplex/ligand solutions are shown in Figure 3.1. Quadruplex ions in the -4, -5, and -6 charge states are observed for Q2 (Figure 3.1a), and the quadruplex/ligand complexes are easily discerned and assigned in Figures 3.1b-d based on the characteristic mass shifts caused by the bound ligand(s). Ligand **1** exhibits the greatest relative extent of binding, whereas ligand **2** engages in very little quadruplex binding. The bulky *tert*-butoxycarbonate (BOC) group of ligand **2** appears to significantly suppress DNA binding. Ligand **3** exhibits lower binding affinity than **1**, and this difference is ascribed to the lack of a third possible hydrogen bonding interaction which is possible for **1**. For the three nucleosides, the relative quadruplex binding affinity follows the trend **1** > **3** > **2** indicating that the improved hydrogen bonding motif and lack of steric interference allows **1** to bind to Q2 more effectively than the other two compounds. A similar trend was observed for binding

to Q1 albeit with greater binding between Q1 and **3** than was seen with Q2 (mass spectral results not shown), and the complete set of comparative results is shown in bar graph form in Figure 3.2 for easy visual comparison. The differences in ligand binding between Q1 and Q2 are most likely due to the different conformations at either end of each quadruplex since the quadruplexes themselves are of the same length. Since Q1 is an intermolecular quadruplex while Q2 is an intramolecular one, the thymidine nucleotides at either end of the quadruplexes will be in different conformations: unstructured for Q1 and in thymidine loops for Q2. This could create different binding pockets for ligand interaction.

3.4.2 Concentration Dependence of Ligand Binding.

To evaluate whether the interaction between **1** and quadruplex DNA is a specific association or a non-specific aggregation, solutions containing Q2 with varying concentrations of **1** were analyzed (Figure 3.3). The concentration of DNA was fixed at 10 μM, and the ligand concentration was varied from 20 to 50 to 100 μM. Over this range, the extent of complexation between **1** and Q2 remained unchanged. The lack of dependence of the extent of complexation on the concentration of **1** offers evidence against non-specific aggregation between **1** and the quadruplex DNA. Analogous concentration-dependent experiments were carried out with **3** and indicated a significant degree of non-specific binding, based on the substantial increase in the stoichiometries of the DNA/ligand complexes as well as the notable increase in the fraction of bound DNA

as the relative concentration of ligand **3** increased (Figure 3.3). For example, up to five molecules of **3** are bound to one quadruplex in Figure 3.3f. The high number of bound

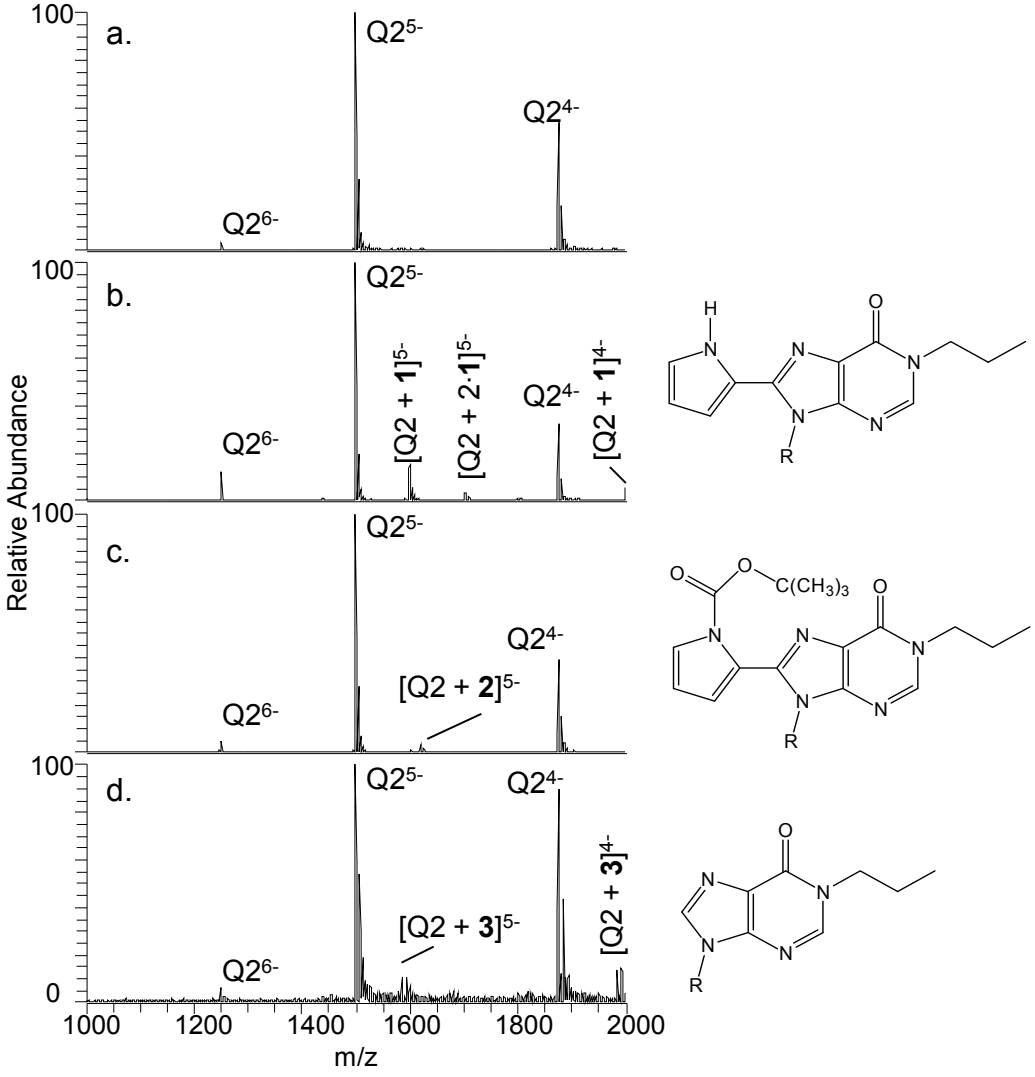


Figure 3.1 ESI-mass spectra of Q2 (10 μM) alone (a) and with **1** (b), **2** (c), or **3** (d) with each ligand at 20 μM. The label Q indicates the quadruplex form. The ligand structures are pictured to the side of the associated spectrum.

ligands suggests that **3** is aggregating with the quadruplex in a non-specific manner, a situation that does not occur for **1**. When analyzed alone, both **1** and **3** show the same extent of dimer and trimer formation indicating that the differences seen in binding are not related to simple ligand aggregation (data not shown). Results of the same experiment performed with **2** showed minimal binding even at ligand:DNA concentrations as high as 10:1, highlighting the impact of steric hindrance on DNA binding (data not shown).

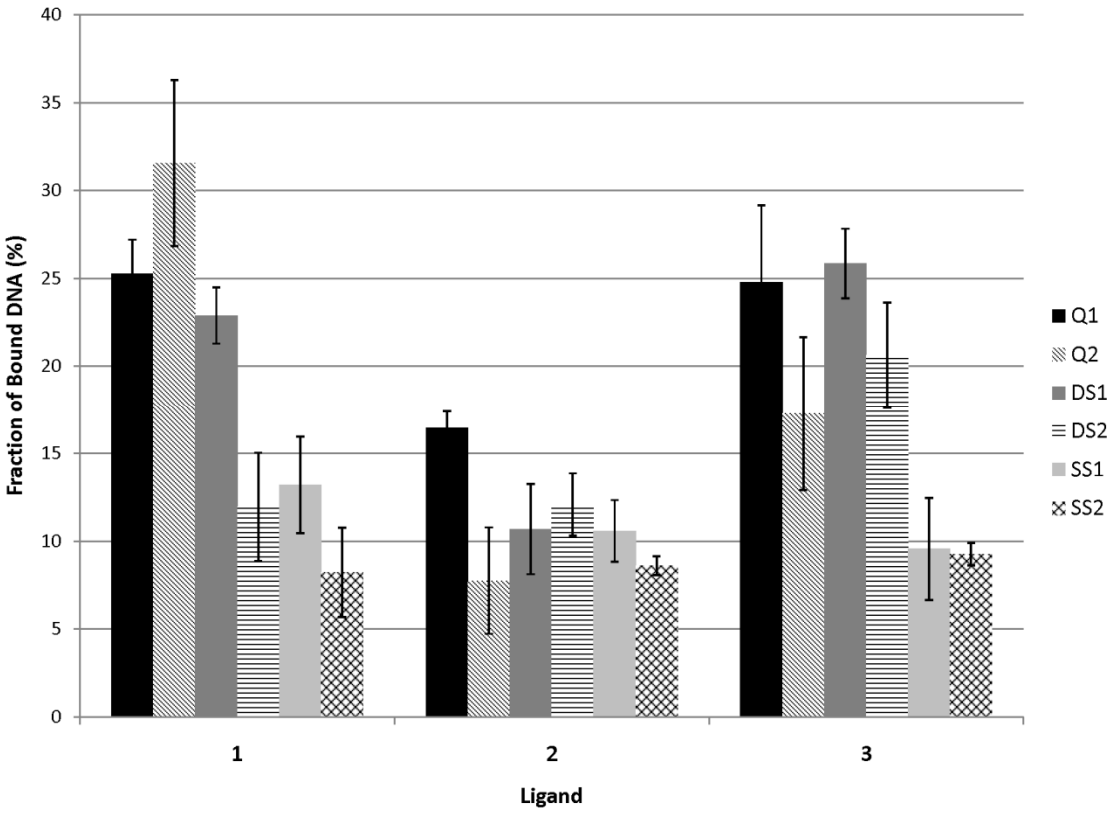


Figure 3.2 Fraction of bound DNA for ligands **1**, **2**, and **3** to quadruplexes, duplexes and single strands. Error bars represent standard deviations after three replicates each. The ratio of ligand to DNA was 2:1 in each case.

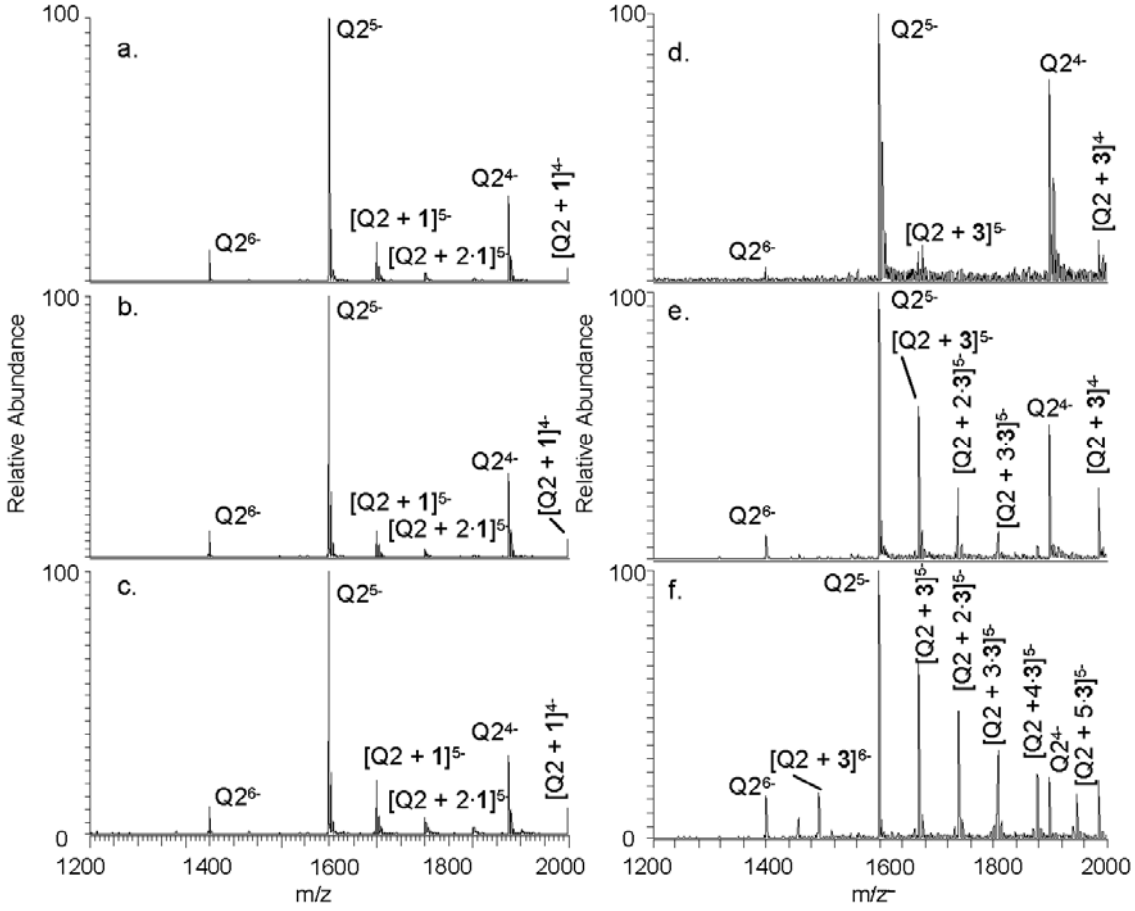


Figure 3.3 ESI-mass spectra of Q2 (10 μM) with varying concentrations of **1** or **3**. Concentrations of **1** were 20 μM (a), 50 μM (b) and 100 μM (c). Concentrations of **3** were 20 μM (d), 50 μM (e), and 100 μM (f).

3.4.3 Interactions with Single-Stranded or Duplex DNA.

To evaluate the selectivity of these ligands towards quadruplex DNA versus other alternative DNA structures, the ligands were incubated with single strand and duplex DNA. The single strands or duplexes were chosen due to their widely different GC versus AT base content. For instance, duplex DS1 contains over 80% GC base composition, whereas DS2 contains only about 25% GC base content. A decrease in

binding of **1** to DS2 is expected if the binding is guanine specific. Examples of the DNA binding results are shown in Figure 3.4 for DS1 with all three ligands. It is evident from comparison of the ESI-mass spectra for solutions containing DS1 versus DS2 (data not shown) that **1** exhibited more substantial binding to the GC rich duplex than the AT rich duplex. In fact, the extent of binding to DS2 was similar to that observed for the individual single strands for ligand **1** (see bar graph comparison in Figure 3.2). This latter result suggests that the binding motif of **1** is G-selective but not necessarily uniquely specific for quadruplex or duplex structures. In the case of **3**, the ligand bound well to both duplexes and with a similar level of binding to both Q1 and Q2, thus indicating a greater degree of non-specificity with this ligand. The bulky BOC group of ligand **2** suppressed binding to both duplexes.

Examples of the ESI-MS binding results involving the single strands are shown in Figure 3.5. Binding was lower for the single strands than for the duplexes or quadruplexes, even for the GC rich single strand. The low level of binding to the single strands as well as the consistency across the three nucleoside ligands suggests that the single strand binding may be due to simple aggregation with no base specificity.

The bar graph shown in Figure 3.2 summarizes the DNA binding trends for the three ligands and the various DNA structures. The binding to guanine-rich oligonucleotides was most significant for **1** which is attributed to its three appropriately-configured hydrogen-bonding sites. As this ligand is designed with a donor-acceptor-acceptor hydrogen-bonding pattern, it is complementary to the acceptor-donor-donor motif of Hoogsteen binding to guanine. However, lack of any evidence of destabilization

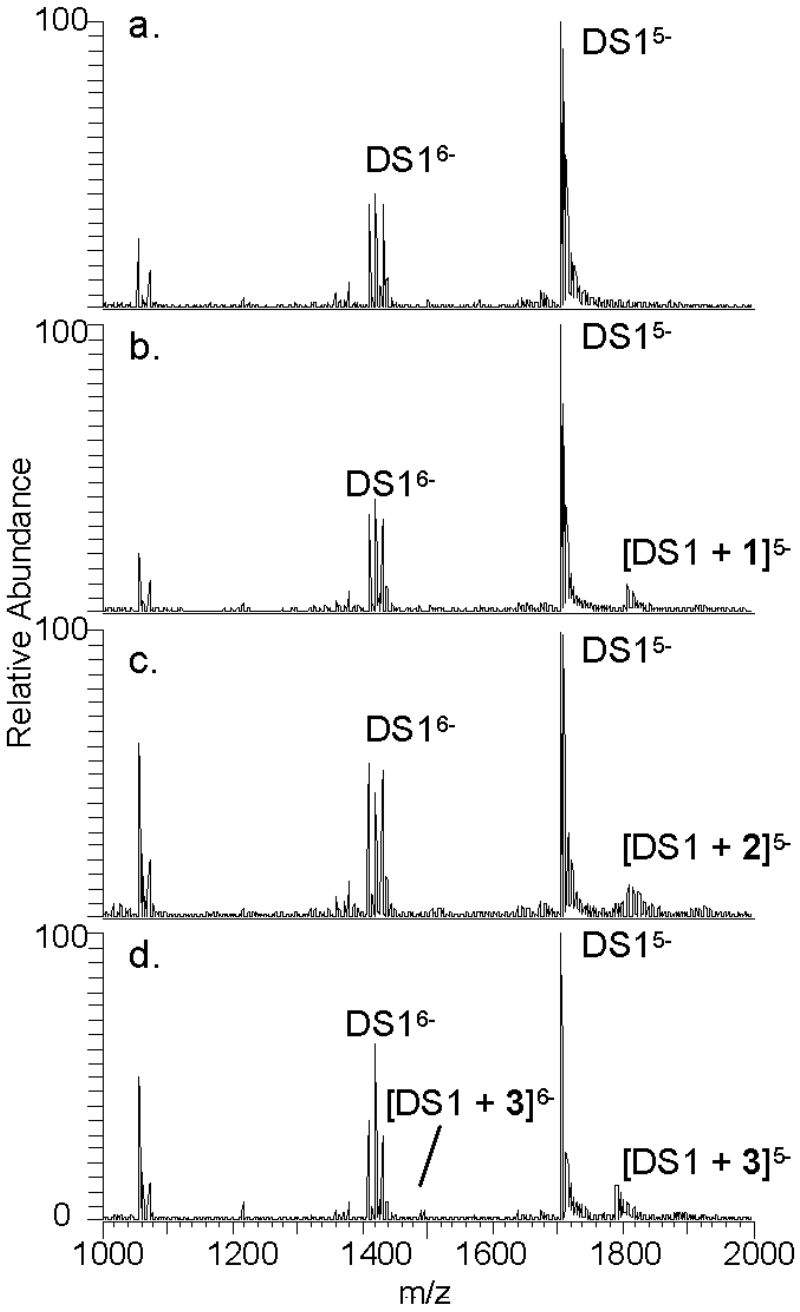


Figure 3.4 ESI-mass spectra of DS1 alone (a) and with **1** (b), **2** (c), or **3** (d). DS indicates the double strand. The duplex concentration was 10 μM and the ligand concentrations were 20 μM each.

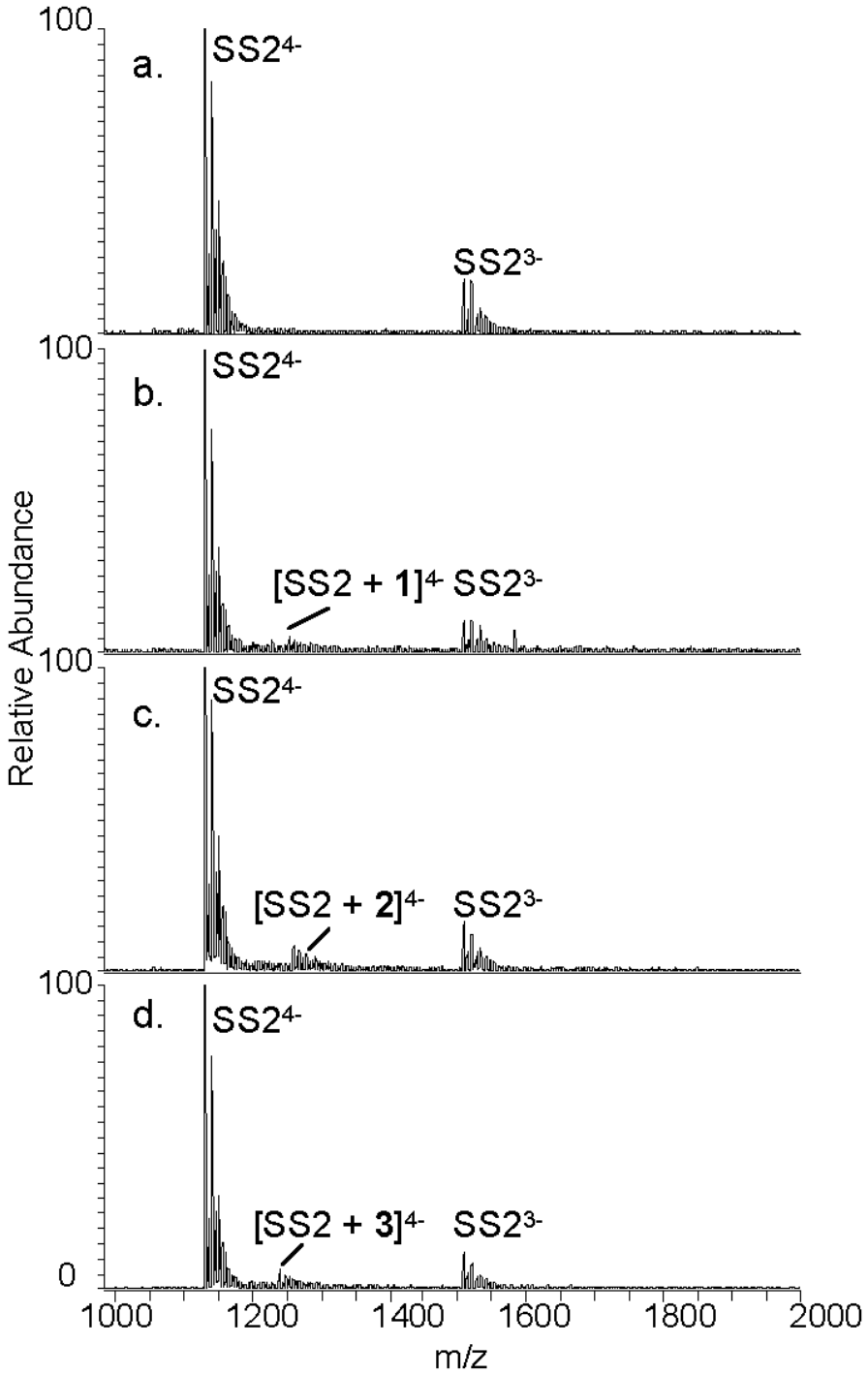


Figure 3.5 ESI-mass spectra of SS2 alone (a) and with **1** (b), **2** (c), and **3** (d). The single strand concentration was 10 μM and the ligand concentrations were 20 μM each.

of the quadruplexes and duplexes upon binding of **1** would indicate that base stacking is a likely binding motif. It is possible the pyrrole functionality enhances the binding to quadruplex and guanine rich duplex structures without disrupting the Hoogsteen or Watson-Crick interactions. For **2**, the single donor hydrogen-bonding site is blocked which, as reflected in Figure 3.2, greatly reduces the ability of **2** to bind to guanine in addition to causing substantial steric hindrance. The steric hindrance factor is alleviated in the third ligand, **3**; however, the lack of a donor hydrogen bonding site reduces its guanine specific binding affinity and makes it a less base-selective structural motif.

3.4.4 Collision Induced Dissociation of DNA/1 Complexes.

The nature of the binding interactions between ligand **1** and DNA was further explored by collision induced dissociation (CID). Previous studies have shown that duplex/ligand complexes which incorporate groove binding interactions dissociate mainly by strand separation and base loss upon collisional activation, with some dependence on charge state[32]. In contrast, elimination of the ligand upon CID conveys that the binding mode is intercalation[32, 33]. Our group and others have also reported different dissociation pathways for quadruplex/ligand complexes based on the binding mode[10, 17, 23]. For quadruplex groove binders, strand separation either with or without base retention has been observed, while end-stacking ligands are typically ejected from the complexes[17, 23]. Dissociation of the Q2/**1** complex resulted in loss of **1** rather than in strand separation (see Figure 3.6a), suggesting end-stacking as a possible binding mode. Dissociation of the -6 charge state of the DS1/**1** complex resulted in loss of the ligand and no strand

separation (see Figure 3.6b). While these CAD spectra cannot definitively confirm the binding mode, these dissociation patterns are consistent with intercalation or end stacking for the duplex and an end-stacking mode for the quadruplex.

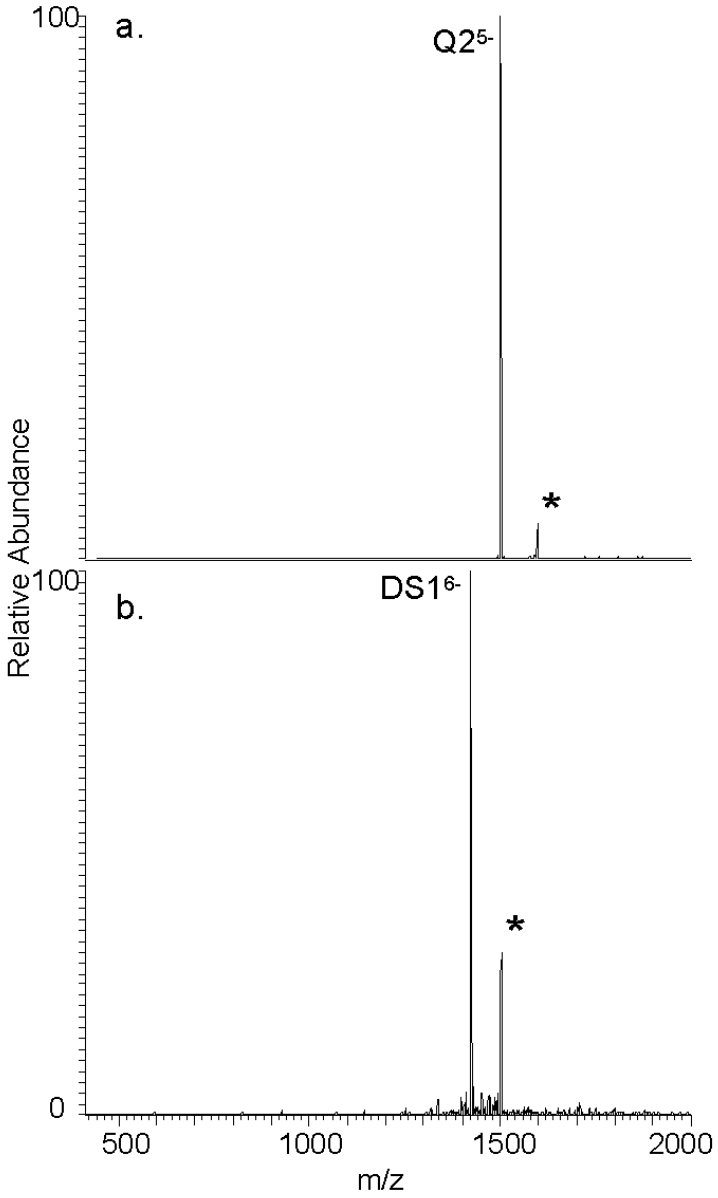


Figure 3.6 ESI-CAD mass spectra for Q2 and DS1. The complexes dissociated were $[Q2 + \mathbf{1}]^{5-}$ (a) and $[DS1 + \mathbf{1}]^{6-}$ (b).

3.5 CONCLUSIONS

Ligand **1** bound two quadruplex structures, $[dTG_4T]_4$ and $d(T_2G_4)_4$, as well as a guanine rich double strand. Ligand **1**, with the capability of forming three-point hydrogen bonds with guanine, was found to have high affinity for guanine-rich sequences, and binding was consistent with specific interactions with guanine bases. The lack of aggregation of **1** with DNA at high concentration ratios supports that the binding is specific. Only low abundance complexes were observed with **2,** thus indicating the impact of steric hindrance caused by the large BOC blocking group. Interactions between the various DNA structures and **3**, especially at high ligand concentrations, indicate that this small ligand binds non-specifically. In previous work undertaken in a predominantly organic solvent, evidence of dissociation of guanine nucleoside clusters upon addition of a water insoluble analog of **1** was interpreted as indicating the ability of this ligand to engage in Hoogsteen interactions that disrupted guanine-guanine binding[28]. Similar results were not explicitly seen in this work suggesting that the binding mode may be more complex. For instance, CID spectra are consistent with end-stacking for the quadruplex/**1** complex and intercalation as a possible binding mode for the DS1/**1** complex, which could explain the apparent guanine selectivity possessed by **1.** The differences in duplex binding between the three nucleosides may be due to the greater number of hydrogen-bonding sites of **1** or simply that the shape is sterically more conducive to intercalation than the others. This work showcases ESI-mass spectrometry as a tool for rapid screening of new synthetic ligands and its role in providing early stage feedback towards the design of base-selective DNA interactive ligands.

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Chapter 4: Evaluation of Binding Selectivities and Affinities of Platinum-Based Quadruplex Interactive Complexes by Electrospray Ionization Mass Spectrometry

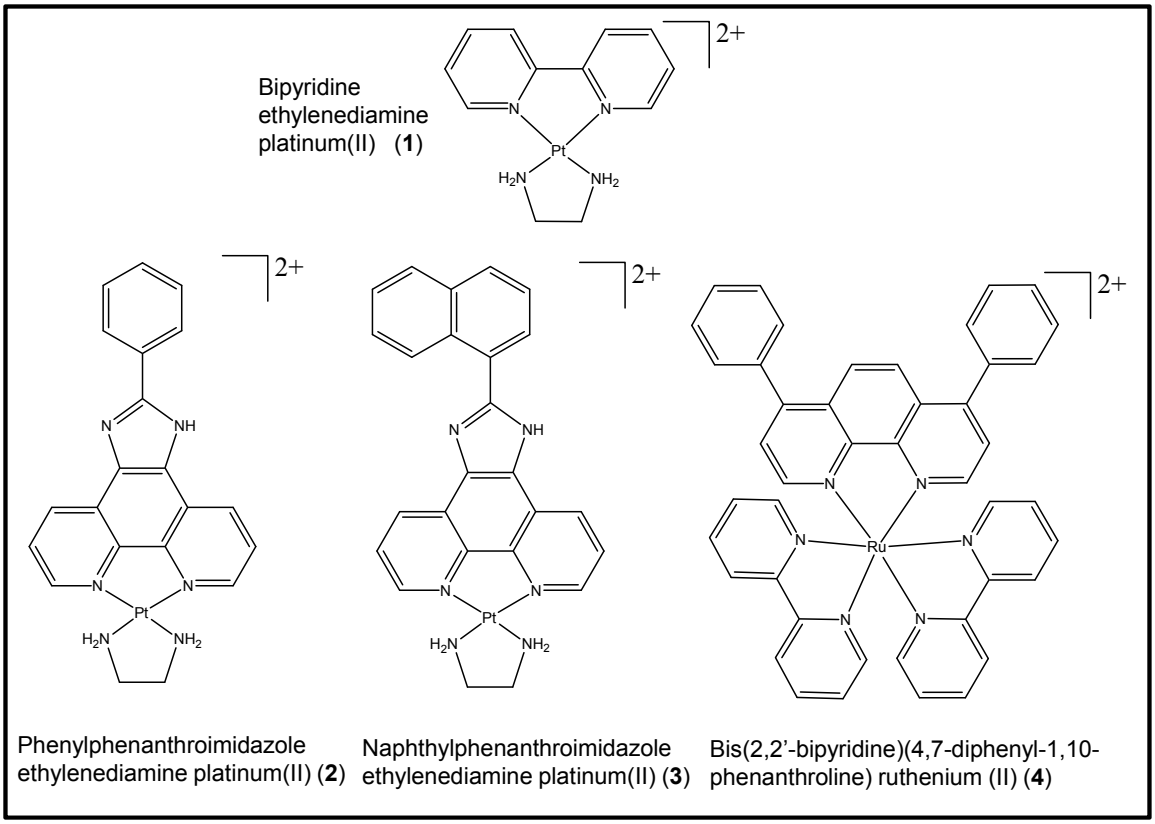
4.1 OVERVIEW

The quadruplex binding affinities and selectivities of two large π-surface Pt^{II} phenanthroimidazole complexes, as well as a smaller π-surface platinum bipyridine complex and a larger Ru^{II} complex, were evaluated by electrospray ionization mass spectrometry. Circular dichroism (CD) spectroscopy was used to determine the structures of various quadruplexes and to study the thermal denaturation of the quadruplexes in the absence and presence of the metal complexes. In addition, chemical probe reactions with glyoxal were used to monitor the changes in the quadruplex conformation due to association with the complexes. The platinum phenanthroimidazole complexes show increased affinity to several of the quadruplexes with elongated loops between guanine repeats. Quadruplexes with shorter loops exhibited insubstantial binding to the transition metal complexes. Likewise binding to duplex and single strand oligonucleotides was low overall. While the ruthenium-based metal complex showed somewhat enhanced quadruplex binding, the Pt^{II} complexes had higher quadruplex affinities and selectivities that are attributed to their square planar geometries. The chemical probe reactions using glyoxal indicated increased reactivity when the platinum phenanthroimidazole complexes were bound to the quadruplexes, thus suggesting a conformational change that alters guanine accessibility.

4.2 INTRODUCTION

Throughout the development of platinum-centered complexes as DNA-interactive agents, the primary focus has traditionally been on covalent attachment rather than non-covalent binding with one landmark example being the anti-cancer drug cisplatin.[1] Other transition metal complexes, such as those based around Ru^{II}, have more often been designed to interact non-covalently with DNA, especially via intercalation.[2-7] However, recent work has focused on creating non-covalent DNA interactive agents with Pt^{II} centers due to the relatively easy synthetic pathways as well as the different coordination geometry of platinum compared to ruthenium which extends the array of possible metal-ligand architectures.[8-10]

The complexes of interest in the present work, phenylphenanthroimidazole ethylenediamine platinum(II) (**2**) and naphthylphenanthroimidazole ethylenediamine platinum(II) (**3**) (see Scheme 4.1), were developed specifically to interact with quadruplex structures. Quadruplexes are a target of considerable biological interest due to their role in regulating the binding of telomerase.[11-16] Telomerase lengthens telomeres, the G-rich sequences at the 3' ends of DNA that allow replication or transcription to continue along the entire length of the strand. When the telomere arranges into a quadruplex structure rather than remaining as an unfolded single strand, telomerase can neither bind nor lengthen the telomere.[15, 17, 18] This prevents cells, more specifically cancer cells, from becoming immortal.



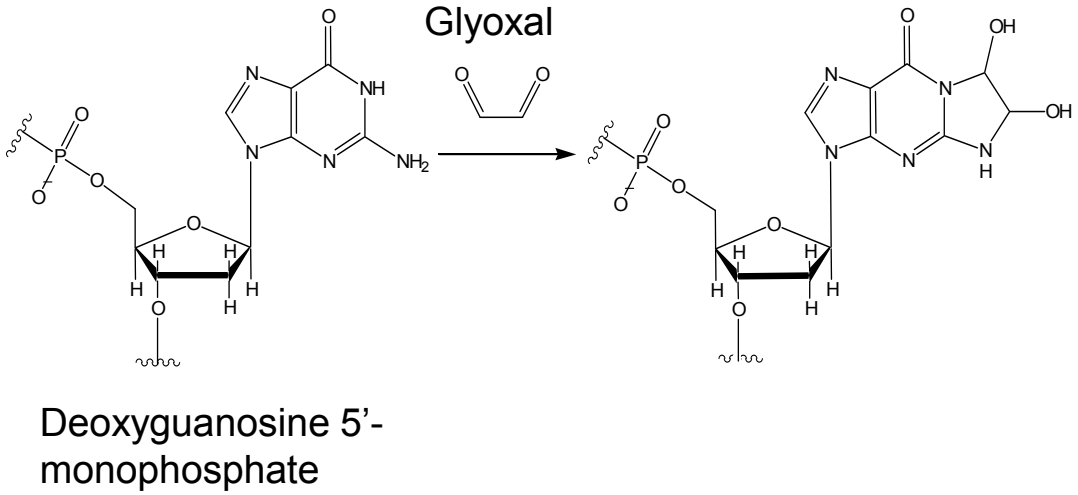
Scheme 4.1. Structures of metal complexes.

In order to increase quadruplex specificity, complexes **2** and **3** were designed with an extended aromatic π-surface area.[19] Since Pt^{II} complexes form a square planar geometry rather than an octahedral geometry, such as that adopted by ruthenium compounds, the π-surface of these transition metal complexes is able to interact more effectively with the planar surface of the G-quadruplex. In comparison to other platinum-based complexes, these complexes have extended π-conjugated surfaces designed for optimal interaction with the G-quadruplex surface through size and electronic complementarity.

In the past decade, electrospray ionization mass spectrometry (ESI-MS) has become a versatile tool for the study of non-covalent interactions. In addition to examination of the interactions between proteins and small molecules or between proteins,[20] mass spectrometry has been very useful for studying the non-covalent binding between oligonucleotides,[21-31] between DNA and proteins,[32-35] between DNA and small molecules,[36-44] and between quadruplexes and ligands.[22, 28, 39, 45-54] Recently, the Ralph group has used ESI-MS and circular dichroism (CD) spectroscopy to probe the binding of ruthenium and platinum complexes to duplex and quadruplex oligonucleotides.[7, 52, 55] It was determined that the small platinum complexes studied showed low binding affinities to quadruplexes in comparison to duplexes.[52] This result is consistent with the prediction that large π-surface areas are necessary to enhance interactions with the G-quartet.

Recently, our group has begun using chemical probes in combination with ESI-MS to study the impact of small molecule binding on oligonucleotide structure.[56] Unlike traditional chemical probe techniques in which the outcomes of the reactions are evaluated using gel electrophoresis, our strategy uses mass spectrometry to monitor the extent of reaction with the chemical probe. In addition, we have applied tandem mass spectrometry, including both collisionally induced dissociation (CID) and infrared multiphoton photodissociation (IRMPD), to determine the identity and location of the chemical probe reaction sites. While our first efforts used potassium permanganate,[57] which oxidizes thymine bases, we are extending our mass spectrometric methods to other DNA-reactive chemical probes, specifically glyoxal in the present study. Glyoxal has been used for traditional chemical probe analysis of both duplexes and quadruplexes.[57-59]

Glyoxal is known to react with guanine bases at the N1 and N2 atoms,[59] as illustrated in Scheme 4.2. As these nitrogen sites are involved in hydrogen bonding between guanine bases in G-quadruplexes, they should exhibit reduced reactivity with glyoxal. Moreover, any change in the secondary structure of a guanine-containing oligonucleotide upon binding of a ligand could change the accessibility of the active sites for reaction with glyoxal.



Scheme 4.2 Reaction between glyoxal and deoxyguanidine.

In this work, the relative DNA affinities of two established duplex intercalators, one a small Pt^{II} complex, **1**, and the other a large Ru^{II} complex, **4**, were compared to two novel complexes based on Pt^{II} centers, **2** and **3** (Scheme 4.1). The latter two complexes have extended structures intended to facilitate binding to quadruplex DNA. While the Ru^{II} complex is similar in size to the novel Pt^{II} complexes evaluated in the present study, the former has an octahedral geometry rather than square planar. The square planar

geometry should increase the π-surface area available for interaction with the quadruplex, thus enhancing binding to the quadruplex. Circular dichroism was used to determine the structure of the quadruplexes as well as to monitor changes in the melting points upon binding of the metal complexes. ESI-MS was used to evaluate the relative binding affinities of the complexes to various oligonucleotide structures. In addition, reactions with glyoxal were undertaken to assess the variation in reactivities of the guanine bases induced by interaction of the complexes with quadruplexes. We present results that show that the novel complexes **2** and **3** have enhanced quadruplex selectivity over the smaller Pt^{II} and Ru^{II} complexes.

4.3 EXPERIMENTAL.

4.3.1 Materials.

Oligonucleotides were obtained from IDT DNA, Inc. (Coralville, IA) and used without further purification (see Table 4.1). Quadruplexes and duplexes were annealed in 150 mM ammonium acetate by placing the strands in a hot water bath and cooling to room temperature overnight (approximately 12 hours). The DNA was annealed at concentrations to produce complexes in the range of 300-500 μM. Thus, DS, Q2, Q3 and Q4 were all annealed at 500 μM while Q1 was annealed at 1.2 mM strand concentration. The annealed solutions were stored for at least 24 hours in the freezer before use. Although the conformations of the quadruplexes obtained for these annealing conditions are well mapped based on CD measurements, these are not necessarily the same

conformations as obtained in vivo. As the SS sequence has the potential to form homodimers or hairpins, non-denaturing gel electrophoresis was used to determine that the majority of the sample is in the single strand conformer under experimental conditions. The Pt^{II} complexes were synthesized as discussed previously.[19] All three complexes were dissolved to 1 mM in DMSO and stored in the refrigerator when not in use. Glyoxal was purchased from Sigma-Aldrich (St. Louis, MO).

Table 4.1 DNA sequences used in this work.

DNA	Sequence	Type of DNA Structure*	Number of Tetrads (for quadruplexes)
Q1	$[d(T_4G_4T_4)]_4$	parallel 4-stranded quadruplex	4
Q2	$dT_4G_4T_4G_4T_4G_4T_4G_4T_4$	antiparallel intramolecular quadruplex	4
Q3	$dT_2G_4T_2G_4T_2G_4T_2G_4$	parallel intramolecular quadruplex	4
Q4	$dT_2AG_3T_2AG_3T_2AG_3T_2AG_3$	parallel intramolecular quadruplex	3
DS	dGCGCGGAACCGCGC/ dGCGCGGTTCCGCGC	duplex	-
SS	dGCGCGGAACCGCGC	single strand	-

*Assuming quadruplexes and duplex annealed in ammonium acetate.

The synthesis of bis(2,2'-bipyridine)(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) was carried out by first synthesizing bis(2,2'-

bipyridine)ruthenium(II) chloride as described previously.[60] One equivalent of 4,7-diphenyl-1,10-phenanthroline was added to this precursor in 70% ethanol, and the mixture was refluxed for 30 minutes.[61] TLC was performed to confirm that only one product was present, and the product was air dried. The identity of the product was confirmed using MS. As with the Pt^{II} complexes, the product (**4**) was dissolved in DMSO and stored in the refrigerator.

4.3.2 Methods.

4.3.2.1 ESI-MS

Three Thermo (San Jose, CA) ion trap mass spectrometers, an LTQ, an LCQ-Duo, and an LCQ Deca modified for IRMPD,[62] were used to perform ESI-MS. For IRMPD, a 50W Synrad CO_2 laser (Mukilteo, WA) was used to irradiate the samples through a hole in the ring electrode. CID experiments were performed on the LCQ Deca instrument using an activation energy of 1.0 V and an activation time of 30 ms. IRMPD experiments employed 99% laser power for 1.3 ms. The ligands and oligonucleotides were combined in a 1:1 ratio, unless otherwise indicated, just before analysis. To assist with desolvation and reduction of salt adducts, 20% methanol and 50 mM ammonium acetate was added to the samples immediately before analysis. The DNA concentration for all ESI-MS samples was 10 μM. ESI-MS was performed in negative mode using a spray voltage of 3.5 kV with a heated capillary temperature of 90^{o}C. The tube lens voltage was varied between -150 and -250 V depending on the particular sample. The percentage of bound DNA was calculated from the ESI mass spectra based on:

$$Percentage\ of\ Bound\ DNA = \frac{RA_{1:1} + RA_{1:2} + \cdots}{RA_{DNA} + RA_{1:1} + RA_{1:2} + \cdots} X\ 100$$

in which $RA_{n:m}$ indicates the relative abundances of the various DNA:metal complex products and RA_{DNA} corresponds to the relative abundance of the unbound DNA in the ESI mass spectra.[40, 44]

4.3.2.2 CD

A Jasco J-815 circular dichroism system (Easton, MD) was used to collect circular dichroism spectra. For melting point determinations, the CD signal, in mdeg, at 293 nm was monitored as the temperature was increased from 25 to 90°C at a rate of 0.2°C/minute. The melting curve was imported into Origin 7.0 (OriginLab, Northampton, MA) for analysis. The melting point, $T_{1/2,}$ was determined by fitting the data with a sigmoidal curve and determining the melting point from the inflection point. Oligonucleotide concentrations for CD were 5 μM.

4.3.2.3 Chemical Probes

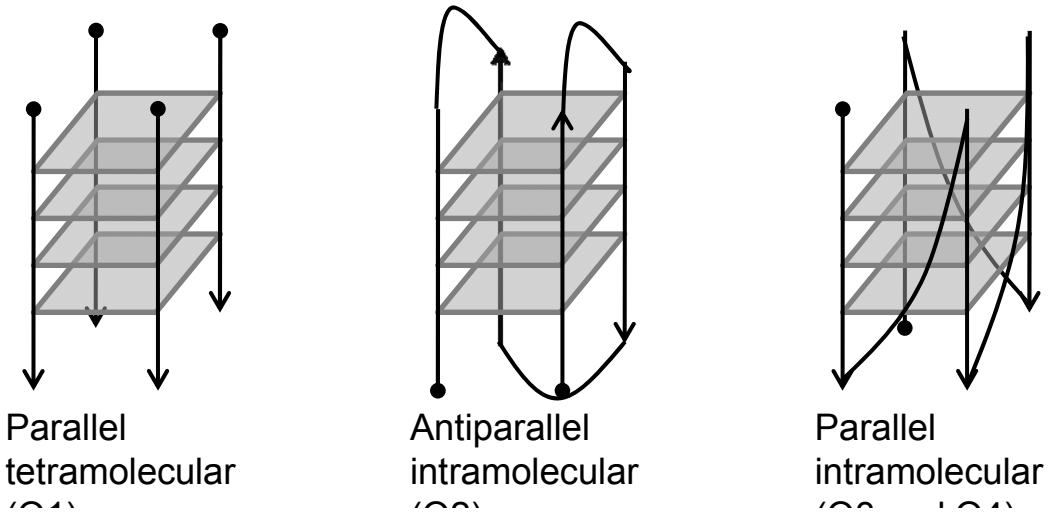
For the glyoxal reactions, 20 μM oligonucleotide complex in water was incubated with 0.5 μL glyoxal for 30 minutes at 37 °C. For the experiments involving the DNA with the metal complexes, the metal complexes were added 10 minutes prior to glyoxal at a 2:1 complex to DNA ratio. The samples were cleaned up using Pepclean C18 spin columns (Pierce, Rockford, IL) to remove excess glyoxal before analysis. These

samples were analyzed by ESI-MS under similar conditions to those described above. Similar calculations to those described above to determine the percentage of bound DNA from ESI mass spectra were used to determine the extent of reaction with glyoxal, but $RA_{n:m}$ indicates the relative abundances of the glyoxal adducts rather than the relative abundances of DNA:metal complexes.

4.4 RESULTS

A variety of experiments in both the gas phase and solution were undertaken to map the nature of the DNA/complex interactions. To determine the degree of structure-specific binding of the metal complexes, a variety of DNA structures were studied. While it was expected that the metal complexes would show preference for quadruplex structures, quadruplexes have a range of orientations and sequences. Four quadruplexes were used in this study: Q1 (a tetramolecular quadruplex), Q2 (an antiparallel intramolecular quadruplex), Q3 (a parallel intramolecular quadruplex), and Q4 (an intramolecular quadruplex with a sequence consistent with the human telomeric repeat) (see representative structures in Scheme 4.3). One guanine-rich duplex, DS, was studied as well, in addition to a guanine-rich single strand, SS, as representative models of other secondary structures. ESI-MS was used to determine the percentage of bound DNA for the various samples to compare the relative binding affinities of the different metal complexes and, when used in conjunction with glyoxal reactions, to reflect changes in the secondary structure of the oligonucleotides upon binding of the metal complexes. While ESI-MS results reveal insight into DNA binding (based on relative abundances and

stoichiometries), CD results give a broader indication of the overall structure of the quadruplex.



Scheme 4.3 Cartoons of quadruplex structures used in this work.

4.4.1 CD spectroscopy of quadruplexes

Quadruplexes have specific CD signatures depending on the overall structure of the quadruplex. For instance, an antiparallel intramolecular quadruplex has a peak at 295 nm and a trough at 260 nm while a parallel quadruplex has a peak at 265 nm and a trough at 240 nm.[63, 64] These features can be used to determine the structures of quadruplexes based on the CD spectra. As expected, Q1 exhibits a peak at 266 nm and a trough at 243 nm (Figure 4.1) which is consistent with a parallel quadruplex. Both Q3 and Q4 yielded similar spectra to the tetramolecular quadruplex (Q1) indicating parallel conformations with positive peaks at 262 nm and troughs at 242 nm. In the presence of sodium or potassium cations rather than ammonium cations (as used in the present study), the

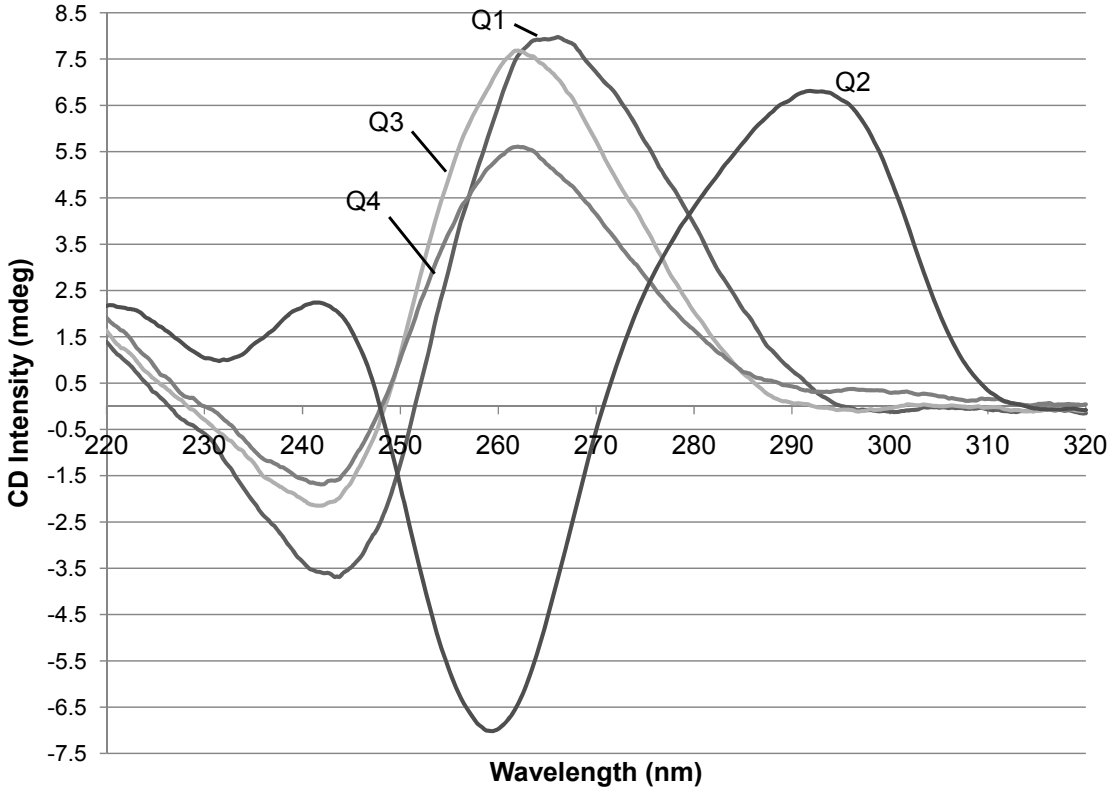


Figure 4.1 CD spectra of quadruplexes after annealing in 150 mM ammonium acetate.

constituent oligonucleotides often result in the formation of antiparallel or mixtures of antiparallel and parallel quadruplexes.[65, 66] In fact, when these sequences were analyzed without annealing, but were mixed with ammonium acetate added immediately before analysis, signature peaks are present for both parallel and antiparallel quadruplexes (data not shown). It is only upon annealing these strands in ammonium acetate that the parallel conformation dominates. The parallel conformation of similar quadruplexes, especially human telomeric sequence (Q4), has been observed with other annealing cations[67, 68] as well as with ammonium.[54] For parallel intramolecular quadruplexes, the loops between

guanine repeats run diagonally across the sides of the quadruplex rather than over the top as is seen with antiparallel conformations.[69] Q2 provided a very different CD spectrum with positive peaks at 292 and 242 nm and a trough at 259 nm. This spectrum is characteristic of an antiparallel quadruplex structure. The longer thymine loops between guanine repeats allows the loops to form over the top of the quadruplex rather than across the sides as occurs with the other two intramolecular quadruplexes. Since complexes **2** and **3** are designed to endstack with quadruplexes, both antiparallel and parallel intramolecular quadruplexes were included in our study to allow evaluation of the impact of the loop structure on the relative DNA binding affinities of the Pt^{II} complexes.

4.4.2 Relative binding affinities and selectivities of metal complexes by ESI-MS

To determine the quadruplex selectivities of the Pt^{II} complexes, solutions containing equimolar concentrations of one of the four complexes and one of the four quadruplexes were analyzed by ESI-MS. Examples of the resulting ESI mass spectra are shown in Figure 4.2, and the results are summarized in bar graph form in Figure 4.3. Based on the ESI-MS results and the calculated percentage bound values, Q2 exhibited very low affinity for the small complex, **1**. The percentage of bound Q2 increased by over an order of magnitude for the two Pt^{II} complexes, **2** and **3**, with **2** more favored than **3**. The Ru^{II} complex, **4**, also showed a high degree of binding to Q2. Interestingly, a comparison of the spectra obtained for Q2 with **2** and **3**, shown in Figures 4.2b and 4.2c respectively, shows that a maximum of two molecules of **3** are bound to the quadruplex

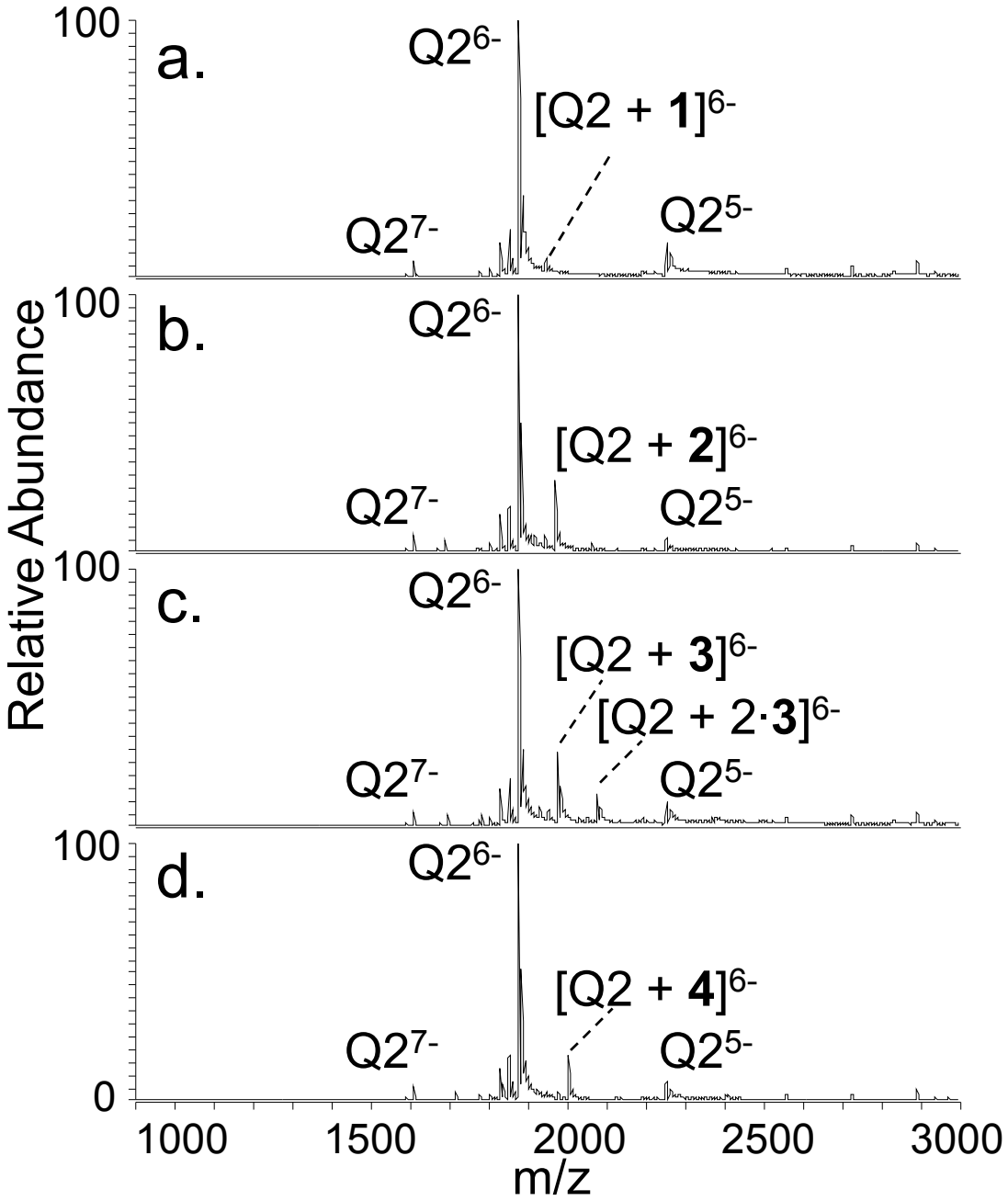


Figure 4.2 ESI mass spectra of quadruplex Q2 with each metal complex. The quadruplex:metal complex concentration ratio was 1:1. The ion naming scheme involving denoting Q2 bound to one moiety of **2**, for example, as [Q2 + **2**] while Q2 bound to two moieties of **2** is labeled [Q2 + 2·**2**].

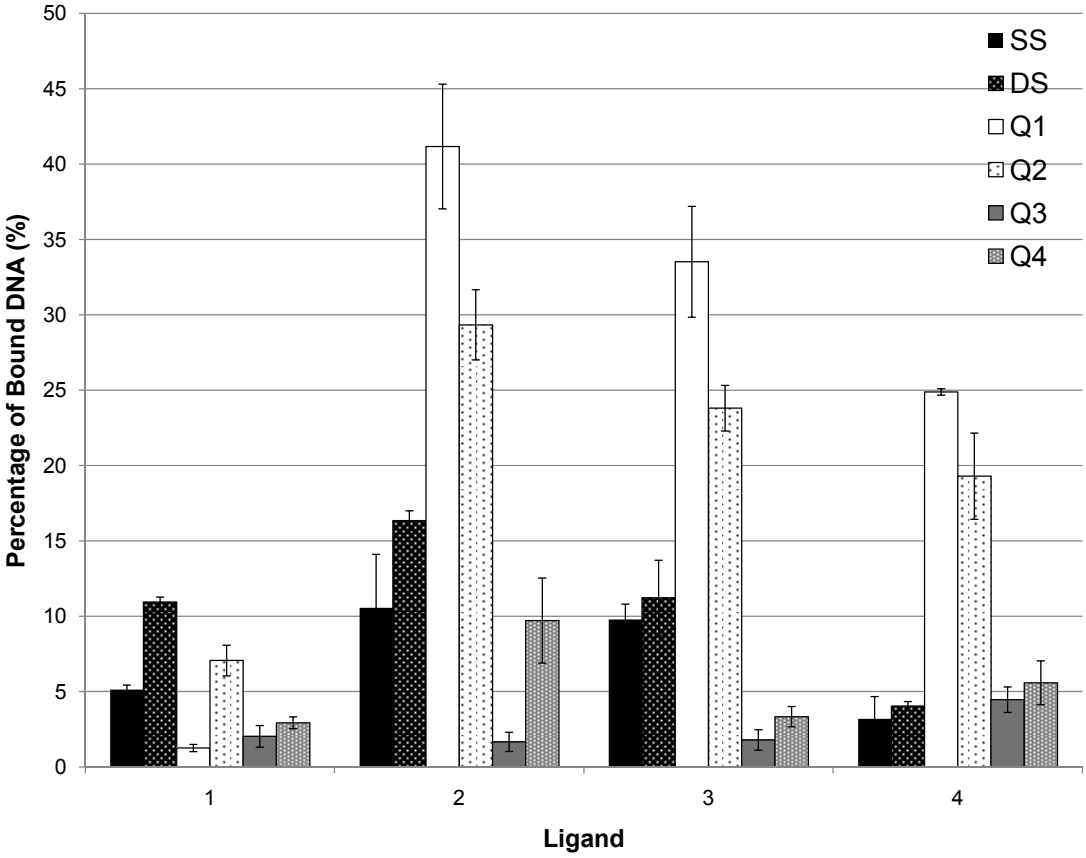


Figure 4.3 Percentage of bound DNA for each metal complex. Each bar represents the average of three samples. Refer to Scheme 4.1 for the structures of compounds **1**-**4**.

while only one molecule of **2** was bound. While it is thought that both Pt^{II} complexes bind through the same mode of interaction, the ability of the larger complex to bind in a 2:1 stoichiometry while the slightly smaller complex only binds in a 1:1 stoichiometry is intriguing and yet unexplained. Both 1:1 and 2:1 stoichiometries are possible for an end-stacking binding mode since there are two ends at which the complexes can bind. Moreover, the small percentage of bound DNA observed for **1** is consistent with the prior results of the Ralph group that showed low binding of small platinum-centered complexes to quadruplexes.[52] Similar trends in binding affinity were obtained for Q1,

which was also used in the previous study reported for these complexes although those results, obtained by UV-vis and equilibrium dialysis, showed greater binding for **3** than for **2**.[19] The abundances of complexes between Q1 and **2** or **3** were far greater than those of Q1 with **1**, with binding to **2** slightly favored.

Surprisingly, quite different results were obtained for the two parallel intramolecular quadruplexes (Q3 and Q4). The percentage of bound DNA for both Q3 and Q4 was found to be 10% or less for each of the four complexes. These results suggest that the geometry of the loop regions with these quadruplexes defines the potential binding interactions with the complexes. The free thymine bases on the ends of the intermolecular quadruplex (Q1) and the arching loops of the antiparallel intramolecular quadruplex (Q2) both facilitate favorable binding between the complexes and the quadruplexes. However, the shorter loops that result in a parallel conformation for the intramolecular quadruplexes (Q3 and Q4) restrict binding with the complexes and result in low abundances for the quadruplex/metal complex species. These loops are suggested to run diagonally across the sides of the quadruplex rather than over the top[69] which may diminish complex binding to the quadruplex. This structure-based suppression of metal complex binding is further supported by the slight increase in binding of **2** to Q4 over Q3 because Q4 has one more nucleotide between guanine repeats which may result in less strained outer tetrads as the extra nucleotide provides greater length to transverse the quadruplex.

To assess the degree of complex selectivity for quadruplexes versus other DNA structures, ESI mass spectra were acquired for solutions containing an equimolar mixture

of a guanine-rich duplex (DS) or guanine-rich single strand (SS) and each of the metal complexes. The resulting mass spectra are shown in Figure 4.4, and the results are summarized in Figure 4.3 to allow easier visual comparison to the quadruplex results. There was minimal binding to the single strand for all complexes and slightly greater binding to the duplex. Complex **1** resulted in a higher percentage of bound DNA for the

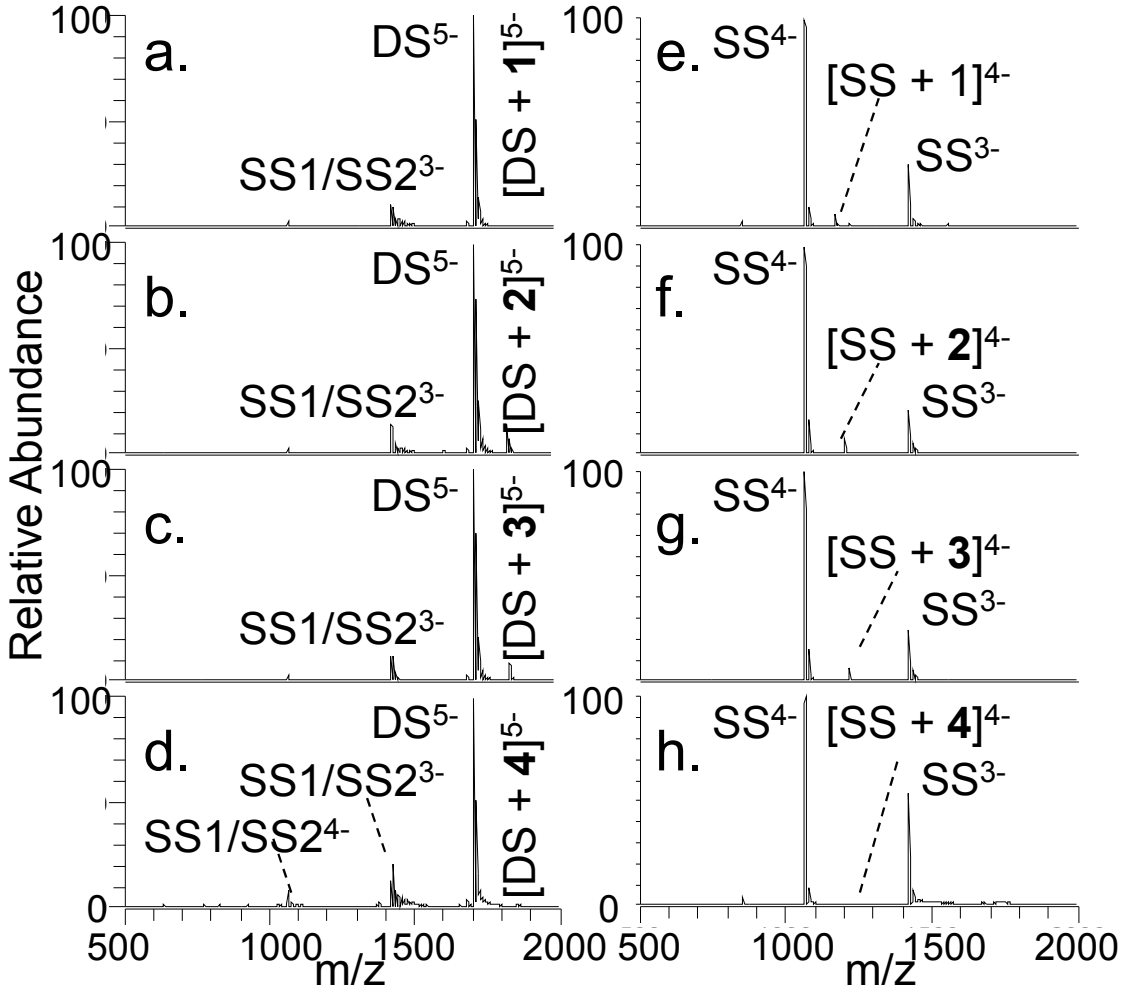


Figure 4.4 ESI mass spectra of DS and SS with each metal complex. The concentration ratio of metal complex to DNA is 1:1.

duplex compared to the four quadruplexes or single strand, thus supporting its greater affinity for duplex DNA. The other three complexes, **2**, **3**, and **4**, all exhibit selectivity for interaction with quadruplexes over the duplex or single strand DNA.

Additional experiments were undertaken in which the concentration ratio of complex to DNA was 2:1 rather than 1:1 (data not shown). While higher concentration ratios were not studied due to salt contaminants in the complexes resulting in uninterpretable mass spectra, the additional complexes present allow more extensive evaluation of the potential stoichometries of each complex with DNA. With SS and DS, in addition to an overall increase in the percentage of bound DNA, **2** and **3** showed low abundances of 2:1 complexes not seen in the spectra acquired with the 1:1 concentration ratio of complex to DNA. Binding with Q3 was similar to that of the 1:1 concentration solutions, with low abundance products with all metal complexes, while the spectrum of **2** with Q4 showed a marked increase in the percentage of bound DNA as well as a low abundance 2:1 product. The greatest changes in product formation were seen for the Q1 and Q2 quadruplexes. The percentage of bound DNA increased and additional higher order products were observed between both of these quadruplexes and **2** and **3**. **2** formed 2:1 complexes with both Q1 and Q2, whereas3:1 complexes were observed with **3** and both Q1 and Q2. The 3:1 complexes of **3** with Q1 and Q2 are surprising since an end-stacking binding mode would only account for association of two molecules of **3** with the quaduplexes. It is possible that the additional **3** is interacting along the side of the quadruplex or two **3** moieties might stack on one another, but there is no specific evidence supporting either hypothesis. When analyzed alone by ESI-MS, **3** did not form

aggregates, but the planarity of the structure might permit stacking. The other metal complexes, **1** and **4**, showed a minimal increase in the percentage of bound DNA for either Q1 or Q2.

4.4.3 Reactions of glyoxal with DNA and DNA/metal complexes

In order to monitor the perturbation of the DNA structures upon non-covalent binding of the metal complexes, chemical probe reactions were undertaken in conjunction with mass spectrometric analysis. As discussed in the experimental section, solutions containing DNA or DNA with one of the metal complexes were incubated with glyoxal, followed by clean-up with C18 spin columns. Glyoxal reacts at guanine bases, thus serving as a chemical probe of accessible guanines.[60] To confirm that thymine bases are unreactive with glyoxal, a 6-mer sequence containing only thymines was reacted under the same conditions as the other strands. The results (not shown) indicate that no reaction occurs. Initial experiments involving the duplexes or quadruplexes in the absence of metal complexes indicated the loss of secondary structure of the DNA after the glyoxal/clean-up procedure based on the detection of only single strand oligonucleotides in the mass spectra. To determine whether this disruption of secondary structure was due to heating during the reactions or to the C18 clean-up procedure, samples of DS and Q1 were analyzed by ESI-MS before heating, after heating at 37^{o} C, and after C18 sample clean up, in each case without the presence of glyoxal. The results (not shown) confirmed survival of the duplexes and quadruplexes both before and after heating. However, after the C18 clean-up procedure, intact quadruplexes or duplexes were no

longer detected by ESI-MS. This series of results indicates that the loss of secondary structure occurs during the C18 clean-up and is not a confounding result of the glyoxal reactions. This means that ESI-MS can be used to monitor the impact of the metal complexes on the DNA reactivity with glyoxal based on detection of glyoxal adducts of the constituent DNA sequences. Typical ESI mass spectra are shown in Figure 4.5.

As expected, SS, with its less rigid secondary structure, proved the most reactive with glyoxal (Figure 4.5a). The guanine bases are not blocked by intramolecular hydrogen bonds or bonds with other guanines or cytosines, thus leaving them more accessible to reaction with glyoxal. Up to four glyoxal moieties were observed bound to the single strand. The reactivity of DS with glyoxal is approximately two times lower than that of SS (data not shown), and a maximum of two glyoxal moieties are bound to the strands. Hydrogen bonding between the guanine bases and complementary cytosine bases apparently suppresses the reactions with glyoxal.

Q1 was chosen for the glyoxal studies because, as an intermolecular quadruplex, the reactions of its constituent sequence (as a single strand) can be studied in parallel to the reactions of the quadruplex. CD spectra of the intramolecular quadruplexes (Q2, Q3, and Q4) indicated that, even without prior annealing, these strands fold into quadruplex structures. Parallel reactions with the single strand conformer permit the separation of the impact of the interstrand hydrogen bonding from that of the metal complexes on glyoxal reactivity, a distinction that would not be possible with an intramolecular quadruplex. To that end, reactions of Q1 and its constituent single strand (Q1ss) with glyoxal were undertaken, and the resulting ESI-MS data are shown in Figures 4.5b and c.

While Q1ss was approximately half as reactive as SS, the latter which contains six guanine bases compared to four guanines in Q1ss, its substantial reactivity with glyoxal confirmed the significant accessibility of its guanines. The reactivity of Q1 with glyoxal

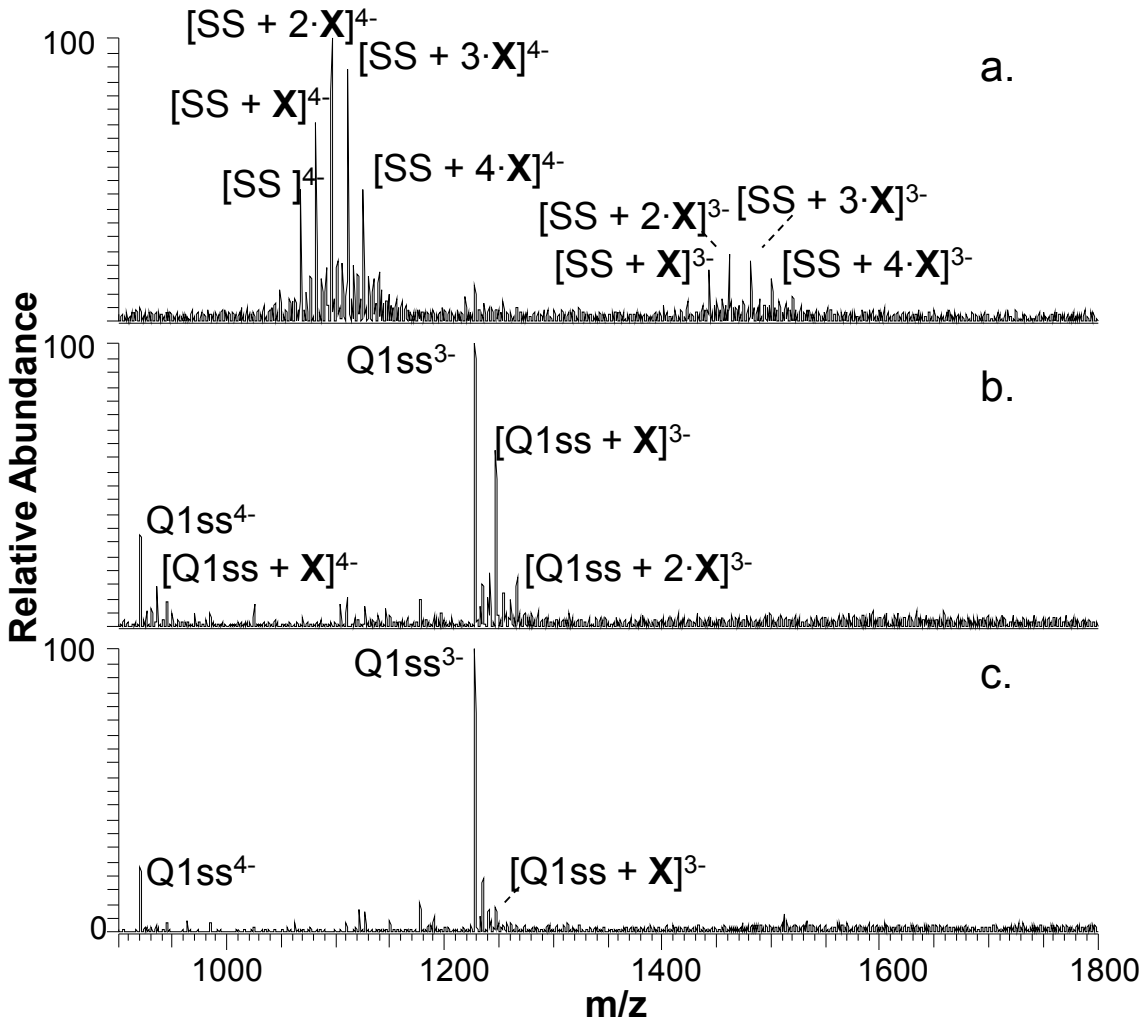


Figure 4.5 ESI mass spectra of DNA after reaction with glyoxal. Spectra were collected for (a) SS, (b) Q1ss, and (c) and Q1. Q1ss corresponds to the single strand form of Q1 that was not annealed. As discussed in the text, the quadruplex Q1 disassembles during C18 clean-up procedure resulting in detection of the single strand Q1ss. Glyoxal adductions are noted by **X**.

decreased by a factor of four relative to that of Q1ss, presumably because the Hoogsteen hydrogen bonds between the guanine bases of Q1 block access to and/or suppress reactions of glyoxal.

Figure 4.6 summarizes the results of similar glyoxal reactions performed after Q1 was incubated with each of the metal complexes. The extent of reaction with Q1 is calculated from the ESI mass spectra in a similar manner to that used to quantify the percentage of bound DNA for solutions containing DNA and the metal complexes. In this case, the abundances of all of the glyoxal-adducted DNA ions were summed and divided by the summed abundances of the all DNA species (both unreacted DNA and the DNA/glyoxal adducts) and then converted into a percentage. It should be noted that the quadruplexes and other non-covalent interactions were disrupted during C18 clean-up of the glyoxal-containing mixtures. The results summarized in Figure 4.6 reflect the extent of reaction based on detection of single strand products even when target DNA was annealed into a quadruplex form prior to incubation with the metal complexes and subsequent reactions with glyoxal. In other words, the extent of reaction of Q1 shown in Figure 4.6 is based on reaction with glyoxal of the same number of guanines as the extent of reaction with Q1ss. Interaction of the quadruplex with **1** caused minimal change in the reactivity of the quadruplex. However, interaction with **2** increased the extent of reaction with glyoxal by a factor of two. This result is an indication that **2** alters the structure of the quadruplex in such a way that increases the accessibility of some of the guanine bases. Similarly, **3** also increases the extent of reaction of the quadruplex with glyoxal, although to a lesser extent than **2**. These data are consistent with the trend in relative

binding affinities discussed earlier in which a higher percentage of bound DNA was found for **2** over **3**. Comparable to **1**, **4** did not cause a change in the extent of reactivity of the quadruplex with glyoxal compared to the free quadruplex. Despite the large size of the ligand, the Ru^{II} complex **4** itself does not appear to cause the same structural changes upon binding that are seen with the Pt^{II} complexes, **2** and **3,** suggesting either a weaker

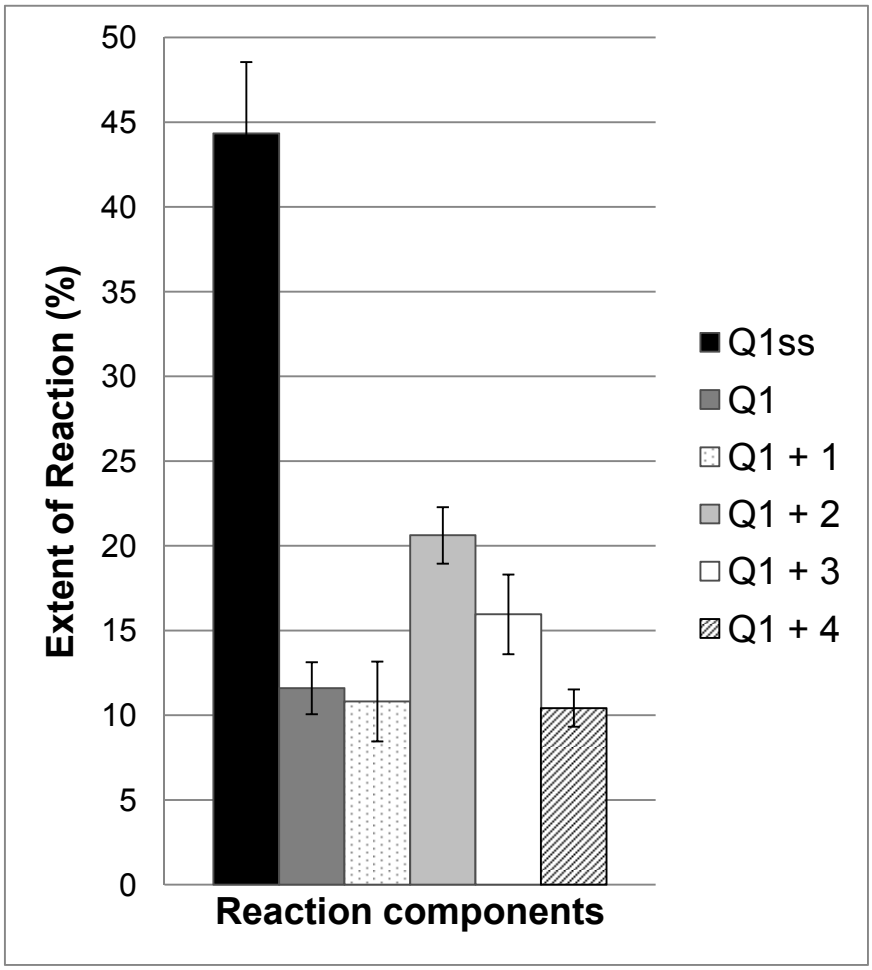


Figure 4.6 Summary of the extent of reaction with glyoxal for Q1ss, Q1 and Q1 in the presence of the metal complexes. The bars represent the average of three reactions.

interaction or different binding mode between **4** and Q1 than **2** or **3**. The possibility of cross-reactivity of glyoxal with the metal complexes themselves, a process that would sequester the complexes and intervene with binding, was also considered. There was no evidence to support the occurrence of this cross-reaction.

The reactions of glyoxal with the double strand, DS, both in the absence and presence of the metal complexes, were also evaluated by ESI-MS (data not shown). Based on the abundances of the DNA/glyoxal adducts, the differences in the reactivity of glyoxal with the free duplex or with the duplex bound to the metal complexes were determined to be negligible. This finding suggests that the metal complexes do not cause a significant change in the structure of the duplex, either in terms of blocking reaction sites or by unwinding the duplex.

4.4.4 Tandem mass spectrometry of glyoxalated quadruplexes

Tandem mass spectrometry via collision induced dissociation (CID) and infrared multiphoton dissociation (IRMPD) was used to examine the structures of the DNA/glyoxal adducts and determine the specificity of the glyoxal reaction sites. Examples of the resulting mass spectra are shown in Figure 4.7 for the Q1ss/glyoxal adducts in the 3- charge state. Upon CID, the most abundant product ion stemmed from loss of the glyoxal moiety (Figure 4.7a). Dehydration of the Q1ss/glyoxal adduct, as well as loss of either a guanine base (e.g. $[M - G]^{3-}$) or a glyoxal-modified guanine base ($M - (G + \text{glyoxal})]^{3-}$), were also dominant fragmentation pathways. These channels are not useful for identifying the specific location of the glyoxal reaction site. Upon IRMPD,

there was more extensive cleavage of the DNA backbone, resulting in both ($a - B$) and w fragment ions that retained the dehydrated glyoxal modification (Figure 4.7b). The majority of these ($a - B$) and w ions stem from cleavages that occur adjacent to guanine bases. Product ions ranging from a_5 to a_8 and w_5 to w_7 were found to retain the dehydrated glyoxal moiety. This array of diagnostic sequence ions indicates that the glyoxal reaction occurs non-specifically at different guanine sites rather than localizing at specific guanine bases. The IRMPD mass spectra acquired from the samples containing

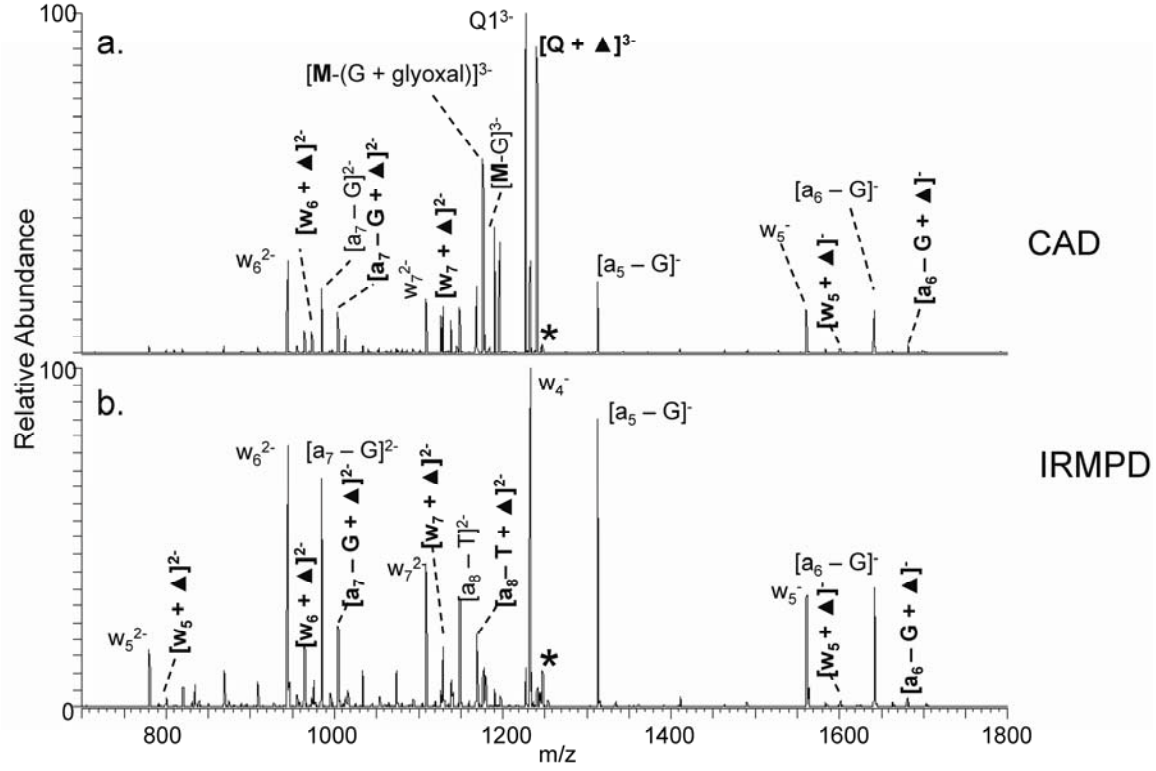


Figure 4.7 Dissociation of [Q1ss + glyoxal]$^{3-}$ by (a) CID at 1.0 V activation energy and (b) IRMPD at 99% laser power for 1.3 ms. Ions that include the dehydrated glyoxal moiety are noted by ▫. The parent ions are indicated by an asterisk.

the quadruplex Q1 in the presence of each of the metal complexes were similar, indicating that the metal complexes do not appear to activate or suppress the glyoxal reaction at uniquely identifiable guanines.

4.4.5 $T_{1/2}$ of Q2 and metal complexes

Relative comparison of quadruplex melting points ($T_{1/2}$ values) in the presence and absence of DNA-interactive ligands can be used to assess how the ligands affect the stabilities of the quadruplexes. An increase in the melting point of a quadruplex upon binding of a ligand suggests an increase in stability against thermally stress. The Q2 quadruplex was chosen for melting point analysis because it produced the most abundant complexes with **2** and **3** by the ESI-MS analysis. In this case, Q2 was analyzed in the presence and absence of the four metal complexes. The melting and cooling curves of the quadruplex did not overlap, indicating the quadruplex was not at equilibrium (data not shown). Although this implies that the melting points are not the true equilibrium melting points and should not be used for thermodynamic calculations, the results are still useful for these simple relative comparisons of thermal stability[70]. In fact, it is common for quadruplexes in general[71] and this sequence in particular[72] to have slow association/dissociation kinetics that result in hysteresis between the melting and annealing curves even at very low heating and cooling rates. Quantitative results are not critical for this work, and the values given for $T_{1/2}$ are useful for relative comparisons.

Without the presence of a metal complex, the Q2 quadruplex yielded a $T_{1/2}$ value of 50.3 ^{o}C (Table 4.2). The addition of **1** caused an insignificant increase in the $T_{1/2}$ (50.5

^{o}C). In addition, **4** only increased the $T_{1/2}$ of Q2 by approximately 4^{o} C (to 54.1 ^{o}C). In comparison, **2** increased the melting temperature 18^{o} C, thus indicating a greater degree of stability against thermal denaturation. Moreover, the addition of **3** caused the greatest increase in the $T_{1/2}$ to 72 ^{o}C. As was shown above, in comparison with **2**, **3** exhibited slightly lower binding affinities to quadruplexes based on the ESI-MS results. However, the protection of Q2 against thermal denaturation by **3** here is greater than **2**. The larger size of the ligand in this metal complex appears to stabilize the quadruplex to a greater extent than **2**, perhaps indicating a higher binding avidity, despite the slightly lower binding affinity estimated from the ESI-MS results. It is also possible that **3** is forming higher order adducts with the DNA than **2**, as is suggested by the ESI-MS results obtained for the 2:1 metal complex:DNA solutions.

Table 4.2 $T_{1/2}$ values for Q2 in the absence and presence of all four metal complexes.

Sample	Melting Point ± Standard Deviation (^{o}C)	$\Delta T_{1/2}$
Q2	50.3 ± 0.2	-
Q2 + **1**	50.5 ± 0.2	0.2
Q2 + **2**	68 ± 1	18
Q2 + **3**	72 ± 1	22
Q2 + **4**	54.1 ± 0.7	3.8

4.5 CONCLUSIONS

The combination of large, planar ligands attached to the Pt^{II} core appears to provide better quadruplex interaction than complexes that have small ligands or are based around a metal with the Ru^{II} octahedral geometry. Thermal denaturation studies and reactions with glyoxal in the presence of the four metal complexes also show that **2** and **3** afford increased thermal stability over the other complexes, as well as induce changes in guanine accessibility in the quadruplexes. While showing relatively high affinities for Q1 and Q2, **2**, **3** and **4** exhibited much lower binding to Q3 and Q4. This striking difference in binding of the complexes highlights the importance of quadruplex structure. As end-stacking binders, these metal complexes are sensitive to the secondary structure surrounding the quadruplex. It should also be reiterated that Q4, in the presence of sodium and potassium, often forms antiparallel or mixed orientation quadruplexes rather than parallel quadruplexes. This could permit binding similar to that seen for Q2 to occur *in vivo*.

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Chapter 5: ESI-MS Characterization of Doxorubicin and Daunorubicin Binding to Mismatches in Hairpin Oligodeoxynucleotides

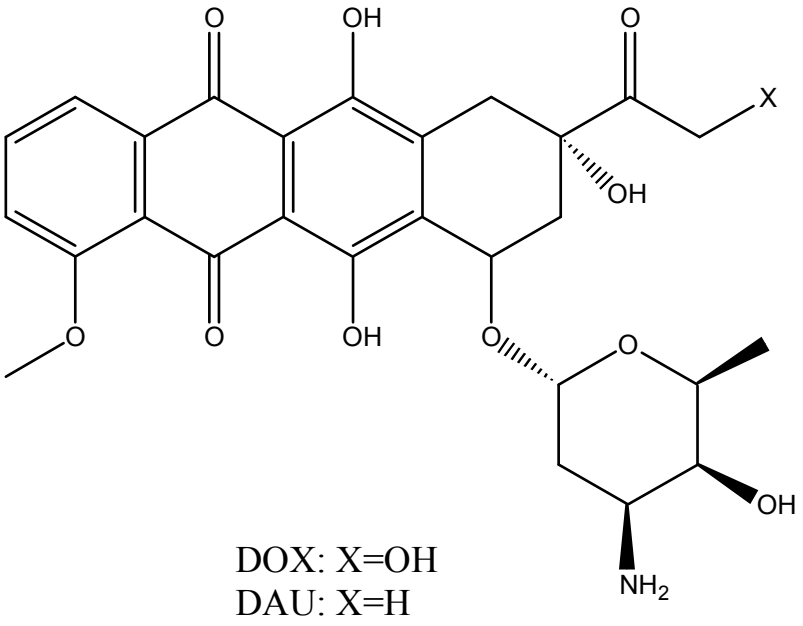
5.1 Overview

Doxorubicin and daunorubicin, non-covalent DNA binding ligands, were studied to determine how binding was affected by the presence of one or more mismatched base pairs. Three hairpin oligodeoxynucleotides were studied: one with a wild type sequence, one with one base pair mismatch, and one with two base pair mismatches. At or near equimolar concentrations, both ligands showed a preference for binding to the mismatched oligodeoxynucleotides.

5.2 Introduction

Electrospray ionization (ESI) allows the transfer of non-covalent complexes from solution to the gas-phase, thus facilitating the analysis of many types of biological complexes by mass spectrometry. This permits the study of duplex[1-3], triplex[4], and quadruplex[4-10] DNA structures, as well as the determination of binding selectivities, stoichiometries, and binding affinities of metal-mediated DNA complexes,[11, 12] DNA/protein complexes[13, 14], and other DNA/ligand complexes.[11, 15-20] In the present work, the complexation of anthracycline ligands, doxorubicin (DOX) and daunorubicin (DAU) (Scheme 5.1), with three hairpin oligodeoxynucleotides was evaluated by ESI-MS. The hairpins used in this work are based on the iron responsive elements (IREs) in mRNA. It has previously been shown that anthracycline ligands bind to IREs[21], and that this binding might be partially responsible for the cardiotoxicity that limits clinical use of

anthracyclines[22]. The hairpins consisted of a wild type control sequence, a second sequence containing a cytosine to thymine mutation, and a third sequence containing two cytosine to thymine mutations. ESI-MS was used to determine the binding specificity of each ligand as well as the stoichiometries of the complexes formed.



DOX: X=OH
DAU: X=H

Scheme 5.1 Structures of anthracycline ligands doxorubicin (DOX) and daunorubicin (DAU).

5.3 EXPERIMENTAL

DOX and DAU were dissolved in DMSO to various stock concentrations (from 10 mM to 100 μM). The hairpins, with sequences shown in Table 5.1, were annealed in BPE buffer by cooling the samples overnight from 90°C to room temperature. Annealed samples were stored in the freezer. Each annealed hairpin (10 μM) was incubated with the desired concentration of the appropriate ligand for 20 minutes in the refrigerator to

Table 5.1 Sequences of oligodeoxynucleotides used in this work.

Name	Sequence
WT	5'-GCCTCCAAATCTTGGAGGC-3'
6	5'-GCCTCTAAATCTTGGAGGC-3'
D	5'-GCCTCTAAATCTTGGAGGC-3'

allow for equilibration, and then 50 mM ammonium acetate was added prior to ESI-MS analysis to assist with desalting. ESI-MS was performed on a Thermo Scientific (San Jose, CA) LTQ-XL linear ion trap mass spectrometer. The source conditions were optimized to minimize disruption of the hairpin/ligand complexes by maintaining the capillary temperature at 90°C and source voltage at 3.5 kV. The tube lens voltage was set to -150 V, and all analyses were performed in the negative mode. The extent of DNA binding is expressed as a "relative binding percentage". For the calculation of relative binding percentages, peak areas were determined using Origin 7.0, and the peak areas of all hairpin/ligand complexes were summed and divided by the summed peak areas of all DNA species as shown in this equation:

$$Relative\ Binding\ (\%) = \frac{PA_{HP/L_1} + PA_{HP/L_2} + \cdots + PA_{HP/L_n}}{PA_{HP} + PA_{HP/L_1} + PA_{HP/L_2} + \cdots + PA_{HP/L_n}} * 100 \qquad \text{(Eq. 5.1)}$$

where PA_{HP} is the peak area of the free hairpin and $PA_{HP/Ln}$ are the peak areas of the complexes containing each hairpin bound to n ligands. Higher values of the relative binding percentages indicate greater hairpin/ligand binding affinities. Three replicates were undertaken for each solution.

5.4 RESULTS AND DISCUSSION

After incubation of each hairpin with each ligand, the resulting solutions were analyzed by ESI-MS. Hairpin/ligand complexes were abundant and easily identified in the resulting ESI-mass spectra. As an example, Figure 5.1 shows an ESI mass spectrum of hairpin WT in the presence of 5 μM DOX (i.e. a 2:1 hairpin: ligand concentration ratio). Complexes containing up to two DOX ligands bound to the hairpin are observed. The oligodeoxynucleotides and complexes tend to have slightly higher levels of salt adduction than are normally observed by ESI-MS due to the presence of the BPE buffer.

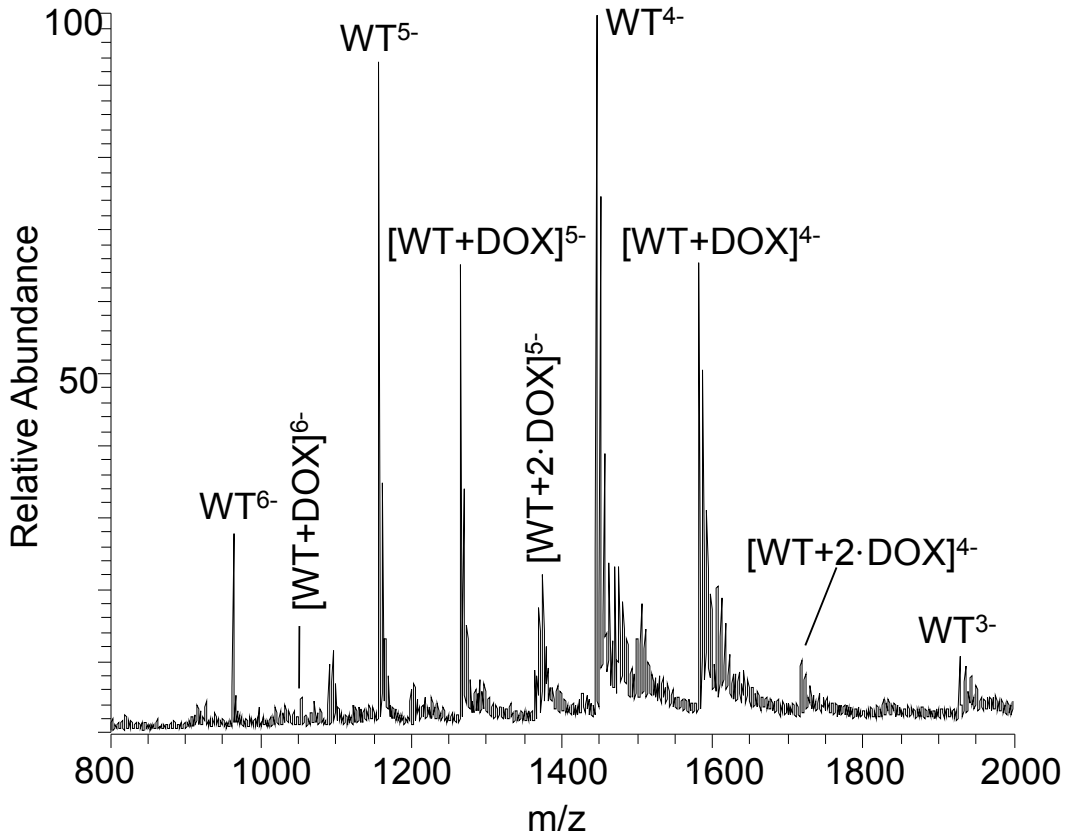


Figure 5.1 Negative mode ESI mass spectrum of WT (10 mM) in solution with DOX (5 mM).

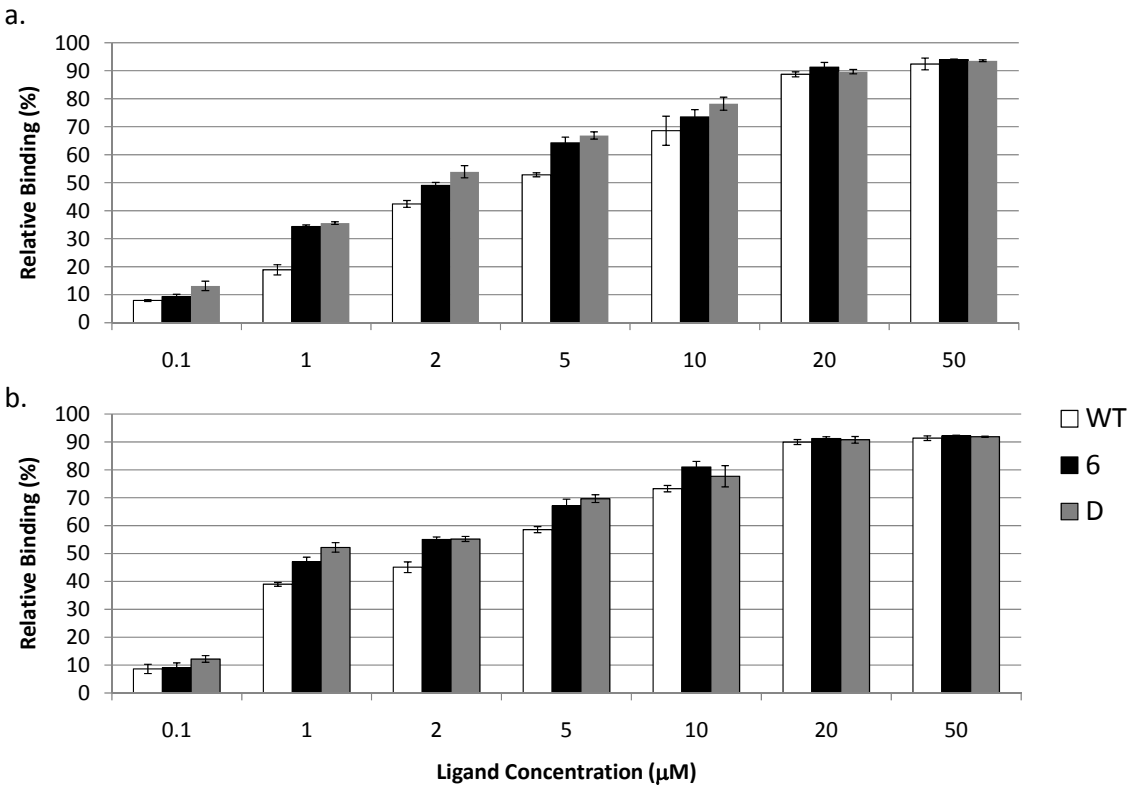


Figure 5.2 Charts comparing relative binding percentages for the three hairpins WT, 6, and D with the ligands DOX (a) and DAU (b).

The relative binding affinities of the ligands to each hairpin were calculated according to Equation 5.1 based on integration of peak areas of the various complexes and unbound hairpin in the ESI mass spectra for each incubate. The results are summarized in Figure 5.2 over a range of ligand concentrations (and a constant hairpin concentration of 10 μM) until virtually saturated at 20 μM ligand. As expected, the relative binding increase percentages increase with increasing ligand concentration. Comparison of the relative binding percentages for DOX concentration ranging from 1 to 10 μM indicate that DOX exhibits greater binding affinities for the mismatched hairpins

6 and D compared to the WT hairpin. The differences were significant to a 95% confidence interval in most cases. For the solutions containing the greater ligand concentrations (20 and 50 μM DOX), the relative binding percentages were similar for all three hairpins, suggesting that there is a significant degree of non-specific binding at higher ligand concentrations. Similar trends were observed for DAU (Figure 5.2b). As with DOX, the binding affinities for the mismatched hairpins 6 and D were greater than that of hairpin WT.

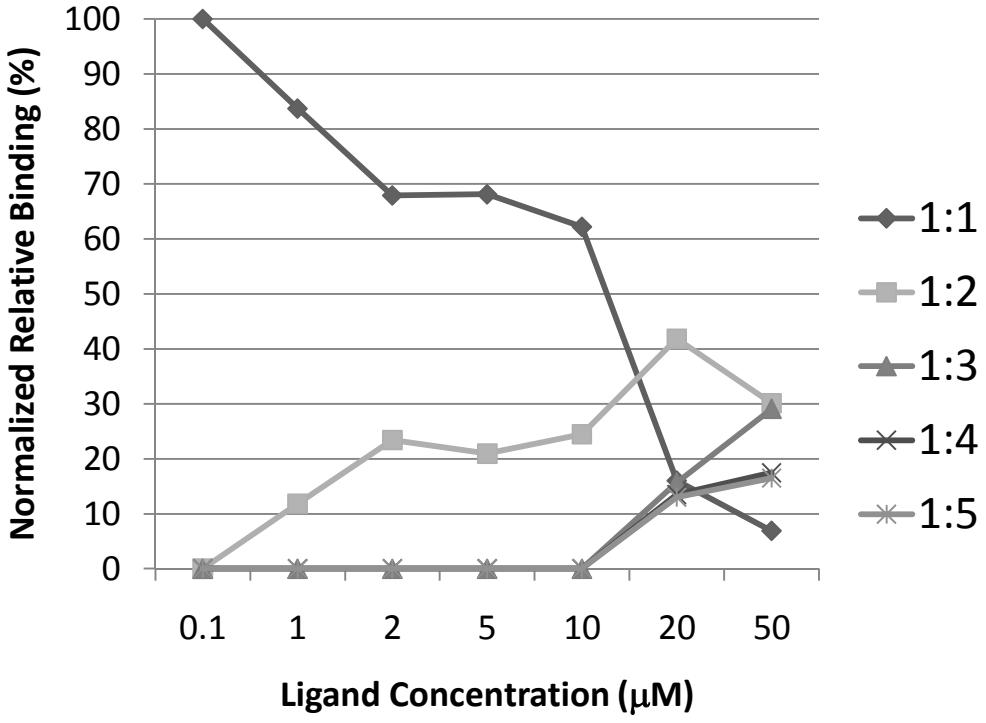


Figure 5.3 Chart of normalized relative binding percentage for each hairpin:ligand stoichiometry for WT bound to DOX.

The stoichiometries of the hairpin/ligand complexes were also evaluated by ESI-MS for solutions containing 10 μM hairpin and 0.1 to 50 μM ligand. The relative

distributions of complexes containing one hairpin and from one to five ligand molecules are plotted in Figure 5.3. At the lowest ligand concentration, only 1:1 complexes were formed. However, once the ligand concentration is increased to 1 μM or greater, 1:2 hairpin:ligand complexes are observed, and even higher order complexes (1:3, 1:4, and 1:5 hairpin:ligand complexes) are detected for solutions containing greater ligand concentrations. The dominance of the 1:1 complexes and to a lesser extent 1:2 complexes is likewise observed for the majority of the hairpin solutions containing WT, 6 or D and either DOX or DAU.

5.5 CONCLUSIONS

ESI-MS allows ready detection of the hairpin/ligand complexes, and comparison of the relative binding percentages confirms that the ligands exhibit higher affinities for the mismatched hairpins than the wild type hairpin. The difference in binding affinities is most notable for solutions containing nearly equimolar concentrations of the hairpin and ligand. At greater ligand concentrations, higher order complexes are observed which signal the occurrence of non-specific interactions that suppress the selective binding of the ligands to the mismatched hairpins.

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Chapter 6: Covalent Cross-links of DNA by RH1 and RH1 Derivatives Studied by LC-IRMPD-MS

6.1 Overview

Two aziridinylbenzoquinone-based DNA cross-linking agents were studied by LC-MS/MS to determine their relative abilities to cross-link oligodeoxynucleotides with different target sequences. RH1 (2,5-diaziridinyl-3-[hydroxymethyl]-6-methyl-1,4-benzoquinone), a clinically studied anti-tumor cross-linking agent was compared to a similar molecule that had been modified with a phenyl group rather than a methyl group. The bulky phenyl substituent was added to determine how steric hindrances would affect the ability to form cross-links in a double helical structure. Cross-links formed by RH1 and PhRH1 (2,5-diaziridinyl-3-[hydroxymethyl]-6-phenyl-1,4-benzoquinone) were observed at 5'-dGNC sites as well as 5'-dGAAC/dGTTC sites. RH1 was more effective at forming cross-links than PhRH1 with all strands. IRMPD and CID results confirmed the presence and the location of the cross-links within the duplexes, and IRMPD was used to identify the dissociation pathway of the cross-linked duplexes.

6.2 Introduction

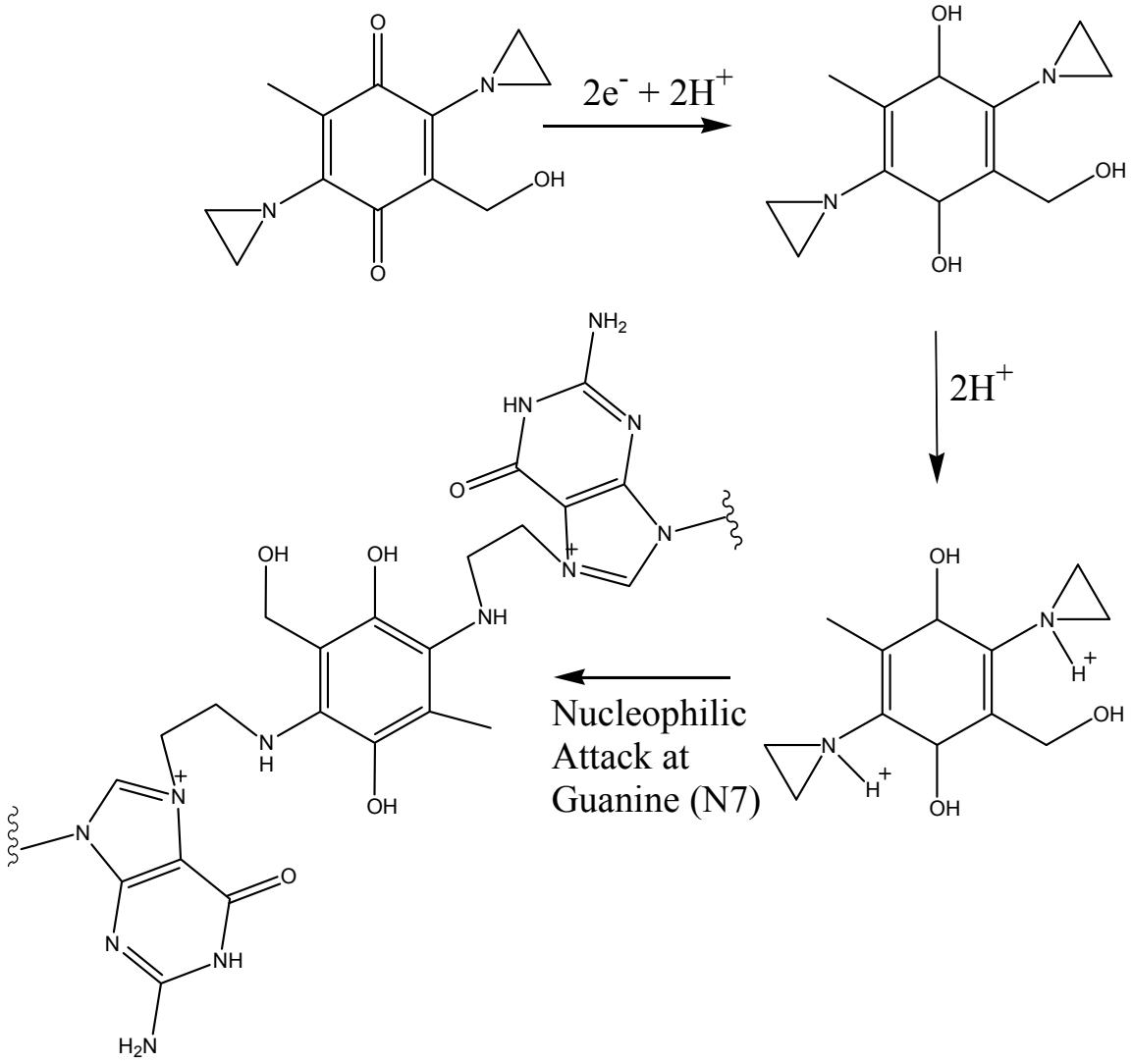
DNA interactive anti-tumor agents can function through non-covalent interactions, such as intercalators[1, 2] and minor groove binders,[3, 4] or through covalent modifications of the nucleobases.[5-9] In many cases, the mechanism of action is to alter the structure of DNA sufficiently that replication machinery does not recognize relevant binding sites, preventing proliferation,[10, 11] or the cellular apoptosis pathway is

triggered.[12, 13] While interstrand cross-links occur relatively infrequently in comparison to monoadducts or intrastrand cross-links, the greatest cytotoxic effects of many anti-tumor agents are thought to be based on the formation of key interstrand cross-links.[14-17] While cross-links can vary greatly in size and position, a major mode of cytotoxicity is the prevention of strand separation during replication.[16]

As anti-tumor agents are designed to prevent cells from replicating, ideally they should exhibit a high level of specificity for tumor cells to prevent widespread death of healthy cells. Bio-reductive agents use the cellular environment of tumor cells to provide specificity.[18-20] Bio-reductive agents enter the cell in an inactive, oxidized form and are reduced by enzymes within the cell to become cytotoxic. This reduction is optimized under the hypoxic conditions which are frequently found in tumor cells.[19] The most well studied of these is mitomycin C which covalently binds two guanine bases after chemical or enzymatic reduction.[21-23] Formation of monoadducts and both interstrand and intrastrand cross-links are possible for mitomycin C with monoadduction being the dominant type of DNA modification.[22, 23] Mitomycin C has been studied extensively and used clinically in the treatment of many cancers including bladder,[24] colorectal,[25, 26] and gastrointestinal,[27] among others.[19, 28-32] Other bioreductive compounds have also been developed including nitroaromatics, aliphatic N-oxides, and heteroaromatic N-oxides.[18] All of these types of compounds are reduced by a variety of cellular reductases before becoming biologically active. The types of interactions between the activated compounds and DNA can range from non-covalent intercalation to cross-link formation.[18]

Another class of bioreductive agent is based on the quinone moiety. For example, benzoquinones with bifunctional mustard moieties have been found to cross-link DNA and the efficiency of the reaction is based upon the other quinone substituents.[33-35] Aziridinylbenzoquinones contain aziridine rings that react with DNA upon conversion of the quinone moiety to the hydroquinone.[36] The reduction to the hydroquinone increases the pK of the aziridine groups, thus allowing the alkylation reaction to occur at physiological pH. Several of these ligands have been found to react with DNA, and the reactivity has been shown to be extremely sensitive to the other substitutions on the quinone.[36-39] One of these agents, RH1, has been studied clinically for its anti-tumor properties.[39] Most recently, it was evaluated for possible use as a chemotherapeutic drug for childhood cancers including neuroblastomas, osteosarcomas, and Ewing's scarcoma.[40] The two aziridine rings allow cross-linking of guanines via the N7 atoms, and the methyl and hydroxymethyl substituents have been found to support cross-linking specifically at 5'-dGNC sequences[36] (Scheme 1). The presence of the cytosine in this segment ensures that this guanine will be in close proximity to a guanine on the opposite strand.

Mass spectrometry has proven to be a versatile tool for characterization of both covalent and non-covalent interactions of ligands with DNA and cross-links in particular. Traditional techniques for analyzing DNA adducts, such as NMR[41, 42] or gel electrophoresis methods,[43, 44] are time consuming and typically have lower sensitivity than mass spectrometry. A variety of DNA binding characteristics can be determined using mass spectrometry. For example, the specificity of binding as well as the binding



Scheme 6.1 Reduction mechanism and possible cross-link structure for RH1. Adapted from ref. 36.

stoichiometries and sequence affinities of DNA interactive ligands can be determined by using ESI-MS.[45-53] In some cases, the binding mode of non-covalent interactions can be determined through tandem mass spectrometry.[54] In addition, chemical probes in conjunction with dissociation techniques can be used to map the sites of DNA/ligand interactions.[55] Ion activation/dissociation techniques can be used to identify the site of

reaction for covalently bound ligands[56] and can also be used to characterize the actual atomic linkages between the ligand and the DNA.[57-59] Coupling tandem mass spectrometry with HPLC as a front-end separation technique, complex samples containing very low levels of covalent cross-links and adducts can be studied.[60-62] These techniques have been used both with small oligodeoxynucleotides[56, 63] as well as with larger, biological DNA samples.[64]

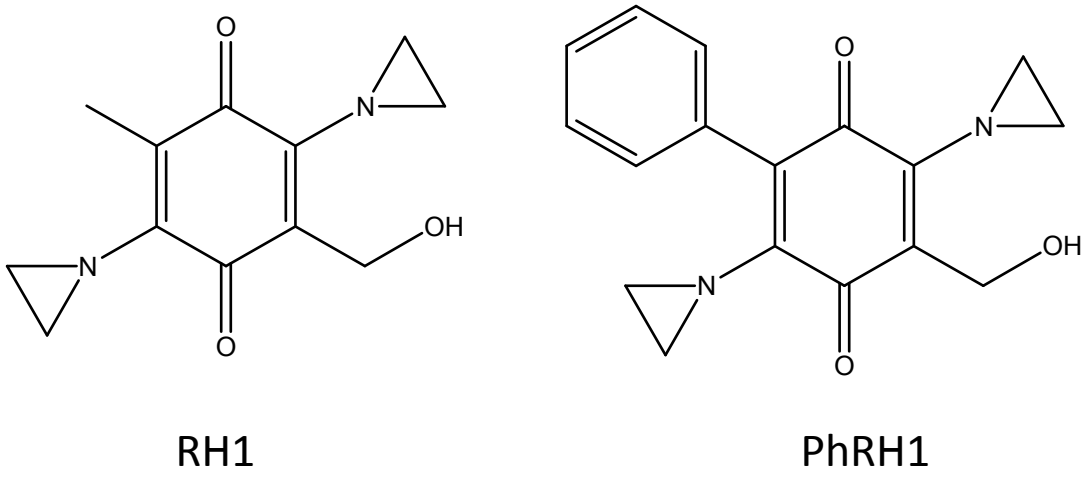


Figure 6.1 Aziridinylbenzoquinones.

In this work, two azirdinylbenzoquinone ligands (Figure 1) will be studied to characterize their cross-linking ability with several duplex oligodeoxynucleotides. The cross-linking efficacy of a phenyl derivative of RH1, PhRH1 in which a methyl group is replaced by a phenyl group, is compared to the cross-linking ability of RH1 to determine the effect of the phenyl substituent on binding and cross-linking. The bulky phenyl group is expected to cause greater steric hindrance to both reduction and to cross-linking which would limit the overall activity. Comparing the abilities of the two ligands to cross-link

various duplexes affords insight into how the quinone substituents modulate the reactivity of the ligands. Further characterization by IRMPD-MS provides information on the location of the cross-links within the oligodeoxynucleotides.

6.3 EXPERIMENTAL

6.3.1 Materials

Oligodeoxynucleotides, as listed in Table 1, were purchased from IDT DNA, Inc. (Coralville, IA) and resuspended in HPLC grade water. RH1 was synthesized as previously described.[39, 65] The phenyl derivative, PhRH1, was custom synthesized. Ammonium acetate, HPLC grade water, and HPLC grade acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). Sodium acetate was purchased from EM Science (Gibbstown, NJ).

6.3.2 Methods

6.3.2.1 Annealing

Duplexes were created from two complementary oligodeoxynucleotides that were annealed in 150 mM ammonium acetate by placing the oligodeoxynucleotides in a 90°C water bath and allowing the two strands to cool overnight to room temperature, over approximately 12 to 14 hours. The annealed duplexes were stored in the freezer.

Table 6.1 Oligonucleotide sequences used in this work

Name	Sequence
DS1	a: 5'-ATATAGCCTATAT-3' b: 3'-TATATCGGATATA-5'
DS2	a: 5'-AAATATGACTATAT-3' b: 3'-TTTATACTGATATA-5'
DS3	a: 5'-AAATTGAACAAATT-3' b: 3'-TTTAACTTGTTTAA-5'
DS4	a: 5'-GACAAATTGACATA-3' b: 3'-CTGTTTAACTGTAT-5'
DS5	a: 5'-TAGACATAGAACAA-3' b: 3'-ATCTGTATCTTGTT-5'
DS6	a: 5'-ATATACGTATAT-3' b: 3'-TATATGCATATA-5'
DS7	a: 5'-AAATATGCATATAT-3' b: 3'-TTTATACGTATATA-5'
DS8	a: 5'-AATTAATTAATTAA-3' b: 3'-TTAATTAATTAATT-5'

6.3.2.2 Reduction/cross-linking reaction

Approximately 2 μmol RH1 or PhRH1 was placed in a conical vial. For RH1, 100 μl of 100 mM sodium acetate (pH 6) was added along with 6.5 nmol of annealed duplex DNA. The same conditions were used for PhRH1, with the exception that PhRH1 was dissolved in 20 μl of DMSO before the addition of the sodium acetate. The solution containing the quinone ligand and the duplex oligonucleotide was placed in an ice bath with argon bubbled through it. In a separate flask, an approximately 0.5 M solution of

sodium hydrosulfite in 100 mM sodium acetate (pH 6) was also kept on ice and under argon. Two 20 μl aliquots of the sodium hydrosulfite solution were added to the quinone/duplex solution 25 minutes apart. Overall, the reaction took fifty minutes. The solution was then exposed to air. Following the reaction, each quinone/duplex solution was desalted using a 3 kDa molecular weight cutoff filter (Amicon Ultra, Millipore, Billerica, MA) followed by a 0.22 μm Durapore filter (Amicon Ultrafree-MC, Millipore, Billerica, MA).

6.3.2.3 LC-MS

The desalted solutions were analyzed by capillary LC-MS. A Dionex (Sunnyvale, CA) Ultimate 3000 capillary HPLC system fitted with a 0.3 X 150 mm Zorbax 300SB-C18 column (5 μM particle size, Agilent, Santa Clara, CA) was used for the separation. The aqueous mobile phase (A) was 10 mM ammonium acetate and the organic mobile phase (B) was 50% 10 mM ammonium acetate/ 50% ACN. The gradient elution method varied depending on the sequences of interest, but the general method involved a gradient from 99% A to 85-80% A over 38 minutes. The flow rate was 4 μl/min. The LC system was directly coupled to a Thermo Scientific (San Jose, CA) LTQ XL linear ion trap mass spectrometer. The mass spectrometer was operated in the negative ESI mode and the capillary temperature was set to 120^{o}C. The system had been modified for IRMPD by the addition of a zinc selenide window at the back of the instrument as described previously.[66] IRMPD was achieved using a Synrad (Mukilteo, WA) 50W CO_2 laser at a

wavelength of 10.6 μm. For these experiments, the laser power was set to approximately 10 W, and the laser was triggered during the activation step for 2 to 10 ms.

6.3.2.4 Relative quantitation

To determine the relative cross-linking efficiencies of the quinones with the duplexes, peak areas were determined from the UV chromatogram. The UV chromatograms were used for relative quantitation to alleviate discrepancies due to variable DNA ionization efficiencies from the results. The peak areas for the cross-linked duplexes were divided by the summed peak areas of all DNA species, including both cross-linked DNA and unmodified single strands, as shown in the following equation

$$Relative\ Crosslinked\ Duplex\ (\%) = \frac{\frac{PA_{CL}}{\varepsilon_a+\epsilon_b}}{\frac{PA_{CL}}{\varepsilon_a+\epsilon_b}+\frac{PA_{ssa}}{\epsilon_a}+\frac{PA_{ssb}}{\epsilon_b}} \quad (1)$$

where PA_{CL} is the peak area of the cross-linked duplex and PA_{ssa} and PA_{ssb} refer to the peak areas of each of the unmodified single strands. Each peak area was normalized with the molar extinction coefficient for the corresponding oligonucleotide sequence, ε_a or ε_b. For the cross-linked duplex, the two extinction coefficients were summed with the effect of the cross-linker ignored. The unmodified intact duplexes did not survive the separation conditions and thus were not observed.

6.3.2.5 UV/vis thermal denaturation curves

DNA melting studies were performed on a Varian Cary Bio 100 UV/vis spectrophotometer (Palo Alto, CA) outfitted with a Peltier temperature control system. The concentration of the duplex was 5μM in 100 mM sodium acetate (pH 6). The duplexes were heated at 1°C/min over a temperature range of 15-80°C. The melting point was determined by finding the maximum of the first derivative curve.

6.4 RESULTS AND DISCUSSION

Both LC-MS and LC-UV methods were used to characterize the reactions between RH1 or PhRH1 and oligodeoxynucleotide duplexes. . The relative abundances of the cross-linked duplexes were compared for different duplexes upon reaction with RH1 and PhRH1, and the fragmentation patterns of the cross-linked duplexes revealed the positions of cross-link formation.

6.4.1 LC-MS detection of cross-links

RH1 was incubated with each selected duplex and reacted under the conditions specified in the experimental section to form the reduced hydroquinone and to encourage the formation of interstrand cross-links. Cross-links were formed between RH1 and five of the duplexes including DS1, DS2, DS3, DS4, and DS5, but not with DS6, DS7, and DS8, as described later. For the five positive cases, the species detected by LC-MS included the unmodified single strands, the cross-linked duplexes, and low abundances of single strand monoadducts. Examples of mass spectra arising from the LC-MS

experiments are shown in Figure 2 and display the multi-charged molecular ion signatures of the single strands, the monoadducts, and the cross-linked duplexes. Intact unmodified (i.e. non-cross-linked) duplexes were not detected. While G/C rich duplexes may remain intact during HPLC separation and ESI-MS analysis, the organic solvent component of the mobile phase disrupts the weaker hydrogen bonding interactions of A/T rich duplexes, thus preventing their survival and detection. In general, the cross-linked duplexes were typically detected in the 5- and 6- charge states.

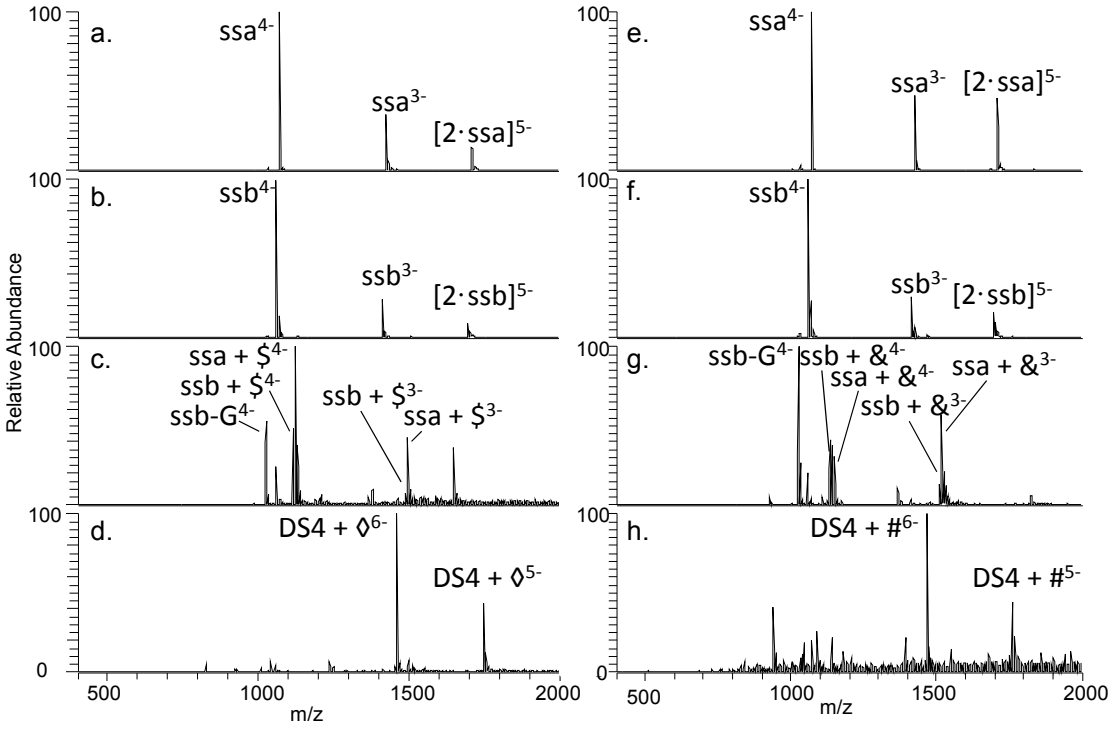


Figure 6.2 Selected mass spectra from LC-MS experiments for RH1 (a-d) and PhRH1 (e-h) after reaction with DS4. Elution times were as follows: a. 19.66-20.4min., b .28.54-29.76 min, c. 33.57-34.43, d. 16.73-17.49 min., e. 19.17-19.73 min., f. 28.88-30.53 min., g. 40.54-41.55, and h. 15.84-16.33 min. RH1 monoadducts are noted by $ and cross-links are noted by ◊ and PhRH1 monoadducts are noted by & and cross-links by #.

Under similar reaction conditions, PhRH1 formed cross-links with the same duplexes as formed with RH1. The PhRH1-cross-linked duplexes tended to elute slightly later than the RH1-cross-linked duplexes. Monoadducted single strands were not observed in high abundances for either RH1 or PhRH1. With many cross-linkers, monoadducts are more abundant than cross-links; however, the reaction mechanism of RH1 and other aziridinylbenzoquinones enhances cross-link formation. After the first aziridine group has opened and has alkylated N7 on the guanine base, an electron pair is donated to the aromatic ring system which increases the reactivity of the remaining aziridine ring improving the yield of the cross-link over the monoadduct.[36]

To compare the reaction efficiencies of RH1 and PhRH1, the relative percentages of cross-linked duplexes were calculated as described above in Equation 1 (Figure 3). Cross-linking of the duplexes by RH1 appeared to be sensitive to the overall sequence of the duplex. The relative cross-linking efficiencies of RH1 with DS1, DS3, and DS4 were all similar (approximately 4%). DS1 and DS4 both have target sequences of 5'-dGNC while DS3 has a target sequence of 5'-dGAAC/5'-dGTTC. DS4 has two cross-linking sites each with 5'-dGAC/5'-dGTC sequences. At first glance, a comparison of the cross-linking yields for DS2, with one 5'-dGAC/5'-dGTC target site, and DS4, with two 5'-dGAC/5'-dGTC target sites, would appear to indicate a linear increase in cross-linking with the number of target sequences. However, MS/MS characterization of the cross-linked DS4, as discussed below, indicates that only one of the two target sites actually undergoes cross-linking. This would suggest that the increase in cross-linking efficiency of DS4 is not due to the increase in the number of target sequences but could be related to

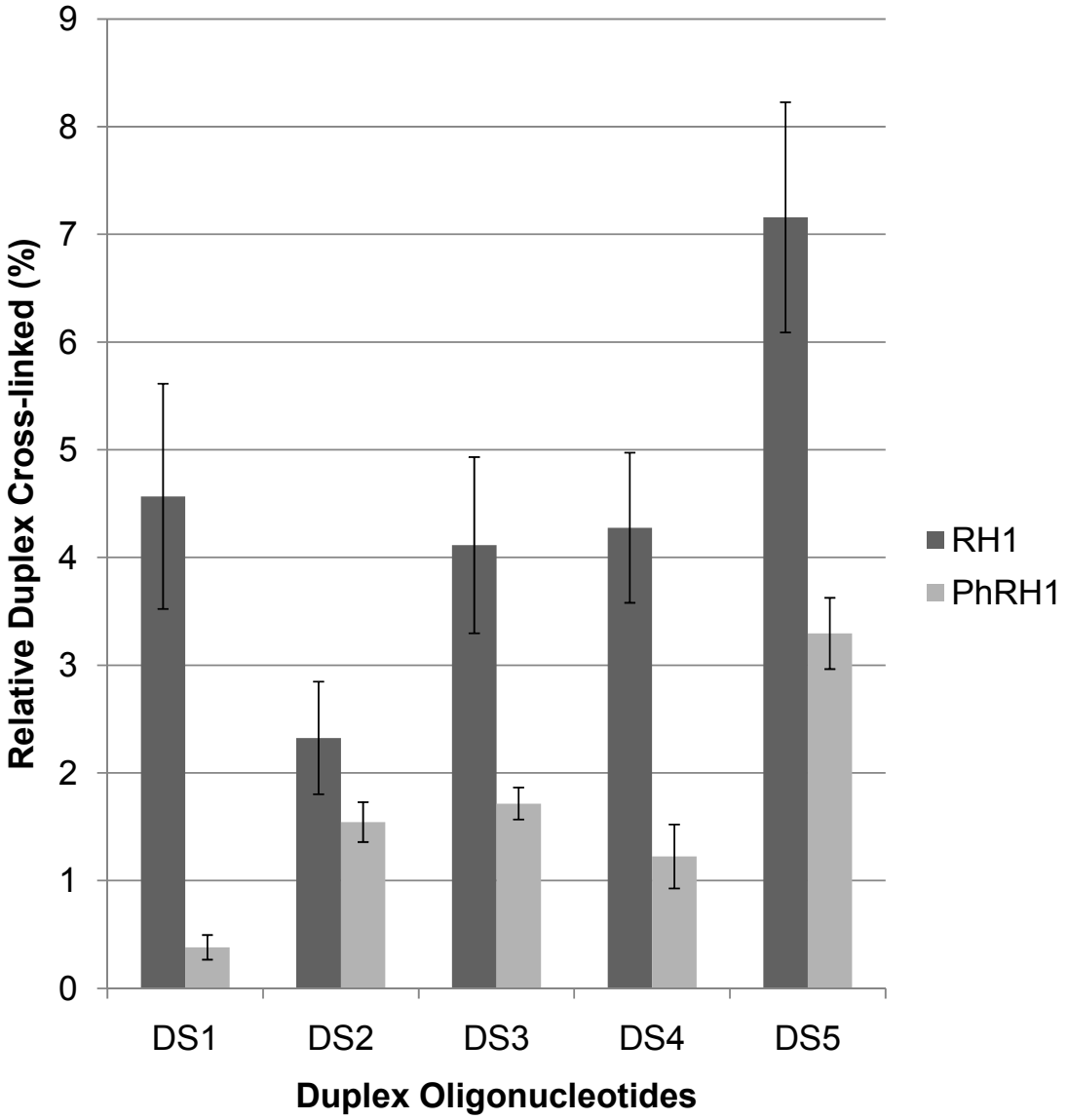


Figure 6.3 Relative percentages of cross-link formation for RH1 and PhRH1.

the change in duplex conformation and stability that occurs when G/C rich sequences are located at the termini of oligodeoxynucleotides. These differences were further explored by determining the melting temperatures of the five duplexes which are discussed in more detail below. The cross-linking efficiency of DS5 was significantly greater than

observed for the other four duplexes. This duplex contains two target sites with 5'-dGAC/5'-dGTC and 5'-dGAAC/5'-dGTTC sequences. The increase in cross-linking, in combination with dissociation results discussed below, indicates that cross-linking may occurr at either target site which therefore increases the probability of cross-linking.

For reactions of PhRH1, the lowest cross-linking efficiency was observed for DS1, indicating that this target site is the least accessible for reaction with the bulky phenyl derivative. Cross-linking of DS2, DS3, and DS4 were generally similar, suggesting that the longer target sequence of DS3 did not provide an increase in flexibility that might enhance PhRH1 cross-linking. As with RH1, the cross-linking of DS5 increased significantly over that of the other four duplexes, and cross-links at both target sites were confirmed by tandem MS.

The cross-linking efficiencies of PhRH1 were lower than RH1 for all five duplexes. The difference in cross-linking efficiency was especially notable for DS1, suggesting that the target site of DS1 is fairly specific for the smaller RH1 and intolerant of the bulkier PhRH1. The difference in cross-linking efficiencies was also significnat for DS3, DS4, and DS5, but the difference was diminished for reactions of RH1 and PhRH1 with DS2. The similarity in cross-linking for DS2 might reflect greater flexibility in the target site which would permit PhRH1 cross-linking to a similar extent as RH1. These results are probed further by examining the melting temperatures of the five duplexes.

To further investigate the underlying reasons for variations in cross-linking by RH1 as a function of the DNA sequences, melting temperatures were measured for the

first five duplexes in Table 1. The melting points spanned the range of 36.1 to 47.9 °C (Table 2), and the order of melting temperatures was DS2 < DS1 < DS3 < DS5 < DS4.

Table 6.2 Melting temperatures of DS1-DS5

Double Strand	**Melting Temperature (°C)[a]**
DS1	38.2 ± 0.2
DS2	36.1 ± 0.4
DS3	41.6 ± 0.3
DS4	47.9 ± 0.3
DS5	44.1 ± 0.4

The sequences with A/T rich terminal sequences, DS1, DS2, and DS3, all have lower melting temperatures than the sequences with G/C bases near the ends which is consistent with the well-known impact of the weaker hydrogen-bonding interactions of A/T versus G/C base pairs. DS2 is the least thermally stable duplex and had the lowest cross-linking efficiency by RH1. In addition, the cross-linking efficiencies obtained for DS2 with PhRH1 or RH1 were very similar- a result not observed for the other duplexes. It is possible that the helix has destabilized and partially distorted, thus preventing RH1 from forming a stable interstrand cross-link. The higher melting temperatures of the other sequences suggest greater stability which would better allow the duplex to remain in a conformation more suitable for cross-linking. DS4 and DS5, which contain the same target sequence as DS2, had melting temperatures approximately 12 and 8°C higher than

DS2, respectively. The additional stability of these two duplexes would allow the duplex to remain in a helical structure conducive to cross-linking efficiency which explains the increased difference in cross-linking between RH1 and PhRH1 in comparison to DS2.

In addition to the duplexes described above, the reactions of RH1 with three other duplexes resulted in no cross-linking although monoadducts were observed with the individual single strands of both DS6 and DS7 which contain guanine residues. The lack of cross-linking to either DS6, a self-complementary 12-mer with a 5'-dCG binding site, or DS7, a 14-mer with a 5'-dGC binding site, suggests that a minimum distance between guanine bases is required to facilitate cross-linking. With the 5'-dCG or 5'-dGC sequences, the guanine bases are on adjacent base pairs- a distance too constricted for optimal bridging by the RH1 ligand. Fragmentation of the monoadducted single strands resulted in loss of the modified guanines suggesting covalent modification. DS8 was chosen to determine if reactions with adenine or thymine were possible in the absence of guanine. No monoadduction or cross-linking was observed for this duplex.

6.4.2 Fragmentation Patterns of Cross-linked Duplexes

The sites of the cross-linking reactions were probed based on the fragmentation patterns generated upon CID or IRMPD of the cross-linked DNA/RH1 ions. Examples of the resulting CID and IRMPD mass spectra are shown in Figure 4 for $(DS3 + RH1)^{6-}$, and similar patterns were observed for all the cross-linked duplexes (in the 6- charge state). CID spectra of the cross-linked duplexes in the 6- charge state generally resulted in fragmentation occurring at the site of cross-linking. For instance, cleavage of the N-

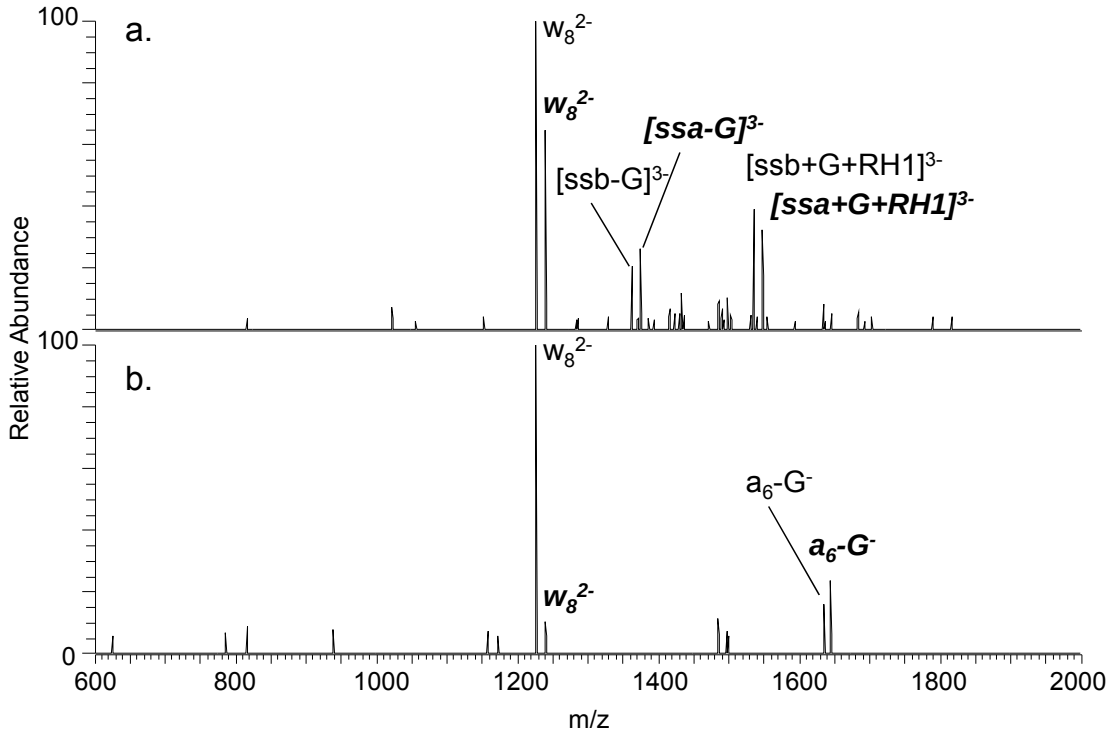
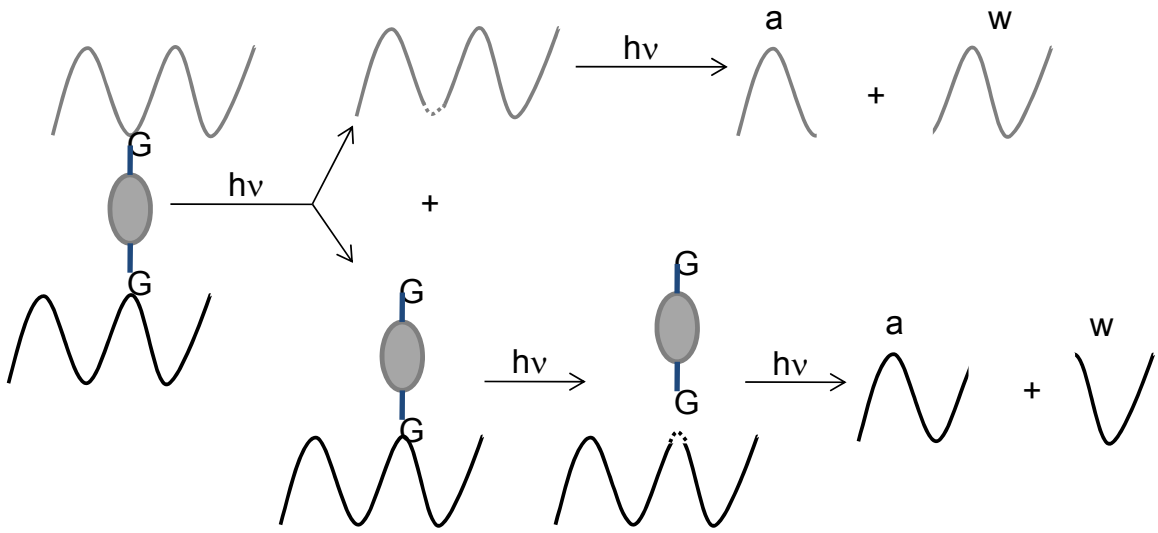


Figure 6.4 CID (a) vs. IRMPD (b) spectra for $[DS3+RH1]^{6-}$. Ssa and ssb refer to the single strands as described in Table 1. The irradiation time for IRMPD was 5 ms. Fragments from ssa are in bold italics.

glycosidic bond between the guanine and deoxyribose sugar resulted in the formation of complementary ions: single strand ions with one guanine lost and single strand ions containing both the cross-linker and an extra guanine (the one lost from the companion strand). A summary of the general fragmentation pathways of the cross-linked duplexes is shown in Scheme 2. The retention of the cross-linker and one guanine from the opposite strand confirms that cross-linking is occurring between the two strands. While these complementary fragment ions are helpful for confirming that interstrand cross-linking has occurred, they do not reveal the specific site of cross-linking. Some backbone



Scheme 6.2 Cartoon of fragmentation process of a duplex with an interstrand cross-link at two guanine bases after IR irradiation.

fragmentation occurs as well, resulting in the production of a - B and w ions. In the example shown in Figure 4, w_8 ions were produced from each strand. This indicates backbone fragmentation 3' to guanine bases in this duplex, but again does not conclusively identify the site of cross-linking because the complementary a - B ions are not present in the spectrum. The 5- charge states of the cross-linked duplexes were also examined by CID. As has been seen with duplexes in low charge states in the past,[67] the major dissociation pathway was loss of unmodified bases. Backbone fragmentation, similar to what was observed upon dissociation of the 6- charge state, was also observed.

As an alternative to conventional CID, IRMPD was also used to characterize the cross-linked duplexes. In IRMPD, ions are energized by absorption of IR photons rather than by a collisional process, typically affording very efficient dissociation with the extent of fragmentation dependent on the irradiation time. For very low irradiation times,

the most abundant product occurred due to the loss of water. Using slightly longer irradiation times (3 ms), CID-like spectra were produced. The dominant fragmentation process involved separation of the strands via cleavage adjacent to a cross-linked guanine site, resulting in formation of complementary single strands, either with a guanine loss or with retention of the RH1 cross-linker plus an extra guanine from the other strand (see Scheme 2). Low abundances of backbone fragment ions, such as w_8^{2-} ions from both strands, were also observed. Increasing the irradiation time to 5 ms promoted secondary dissociation of the single strand species described above, yielding backbone fragment ions as shown in Figure 4. For example, both the pair of w_8^{2-} ions and the complementary a_6 - G ions are observed, in this case confirming the site of cross-linking at those guanines as illustrated in Scheme 2.

CID and IRMPD spectra were also obtained for the cross-linked DS1 and DS2 products (spectra not shown). As with DS3, fragmentation was observed at the site of cross-linking with the primary cleavage occuring at the N-glycosidic bond between the modified guanine and the sugar. Backbone fragments indicating fragmentation 3' to the cross-linked nucleosides were observed for both cross-linked duplexes.

IRMPD was especially useful for characterizing the cross-linked DS4 duplex (Figure 5a). There are two potential cross-linking sites, and it was unclear whether a terminal target sequence would have the correct secondary structure to permit cross-linking. The IRMPD mass spectrum of the RH1 cross-linked duplex in the 6- charge state after 5 ms of irradiation time shows the $(a_4 - G)^-$ ion from ssb as the most abundant fragment. The corresponding w_{10}^{2-} ion is also observed. Fragments from the other strand

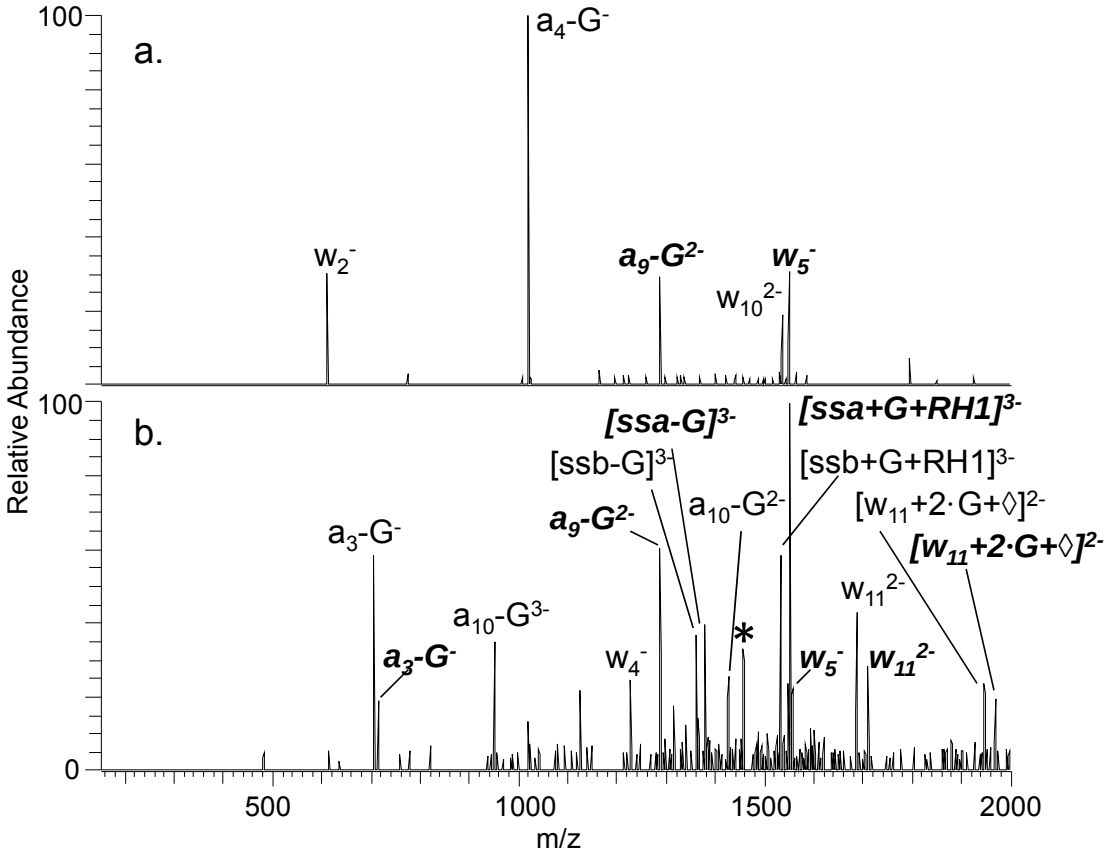


Figure 6.5 . IRMPD spectra for selected cross-linked duplexes. Dissociation spectrum from $[DS4+RH1]^{6-}$ after 5 ms irradiation time (a) and dissociation spectrum from $[DS5+RH1]^{6-}$ after 3 ms irradiation time (b). Fragments from ssa are in bold italics and the diamond indicates the presence of the cross-linker.

include the $(a_9 - G)^{2-}$ ion as well as the complementary w_5^- ion. The significant abundance of these fragment ions, as well as the presence of both sets of complementary fragment ions indicates that the majority of the cross-linking occurred at the internal target sequence. There is a low abundance fragment ion corresponding to the w_2^- fragment from strand b. This could indicate cross-linking at the terminal guanine, but there are no complementary fragment ions observed such as the $(a_{12}\text{-}G)^{2-}$ - a fragment ion

which would be within the m/z range of the instrument. This suggests that the fragmentation at that base is not related to cross-linking.

As with DS4, there were two possible sites of cross-linking for duplex DS5: a 5'-dGAC/5'-dGTC sequence and a 5'-dGAAC/5'-dGTTC sequence. A duplex with two different binding sites that vary in the distance between the guanines was chosen to determine if a cross-linking preference would be seen between the two target sequences. As shown in Figure 5b, the IRMPD spectrum acquired after 3 ms irradiation displays the single strand-type fragment ions described earlier (both single strands with guanine loss and single strands with the additions of the cross-linker and the guanine from the opposite strand). This indicates that cross-links are present but does not indicate to which guanine the cross-linkers were attached. Backbone fragments from strand a present in the dissociation spectrum consist of the a_3 - G and w_{11}^{2-} ions and the $(a_9 - G)^{2-}$ and w_5^- ions. Strand b fragment ions are a_3 - G and w_{11}^{2-} ions and the $(a_{10} - G)^{2-}$, $(a_{10} - G)^{3-}$ and w_4^- ions. These sets of complementary fragment ions correspond to fragmentation at both possible cross-linking sites, indicating that both cross-linking sites are active. In addition to the complementary fragments, two backbone fragments containing the cross-link were observed, including w_{11} ions from each strand with each retaining the cross-linker RH1 and an extra guanine. The w_{11} ion usually does not retain the neighboring base (from the same strand) after fragmentation, but the ions are relatively minor overall. The addition of the cross-linked guanines to the w_{11} ions indicate that, for these particular ions, the cross-link occurred at the 5' guanine on each strand. The presence of this ion from each

strand is a further indication that both sites, the 5'-GNC and the 5'-GNNC sites, participate in interstrand cross-linking.

To map the fragmentation trends and genealogies in more detail, time-variable IRMPD experiments were undertaken. For this strategy, the irradiation time is varied to alter the total energy deposition as well as the secondary activation of primary product ions. The resulting graph obtained for $[DS4 + RH1]^{6-}$ ion is shown in Figure 6. The time-variables curves for the other cross-linked duplexes were similar. As the irradiation

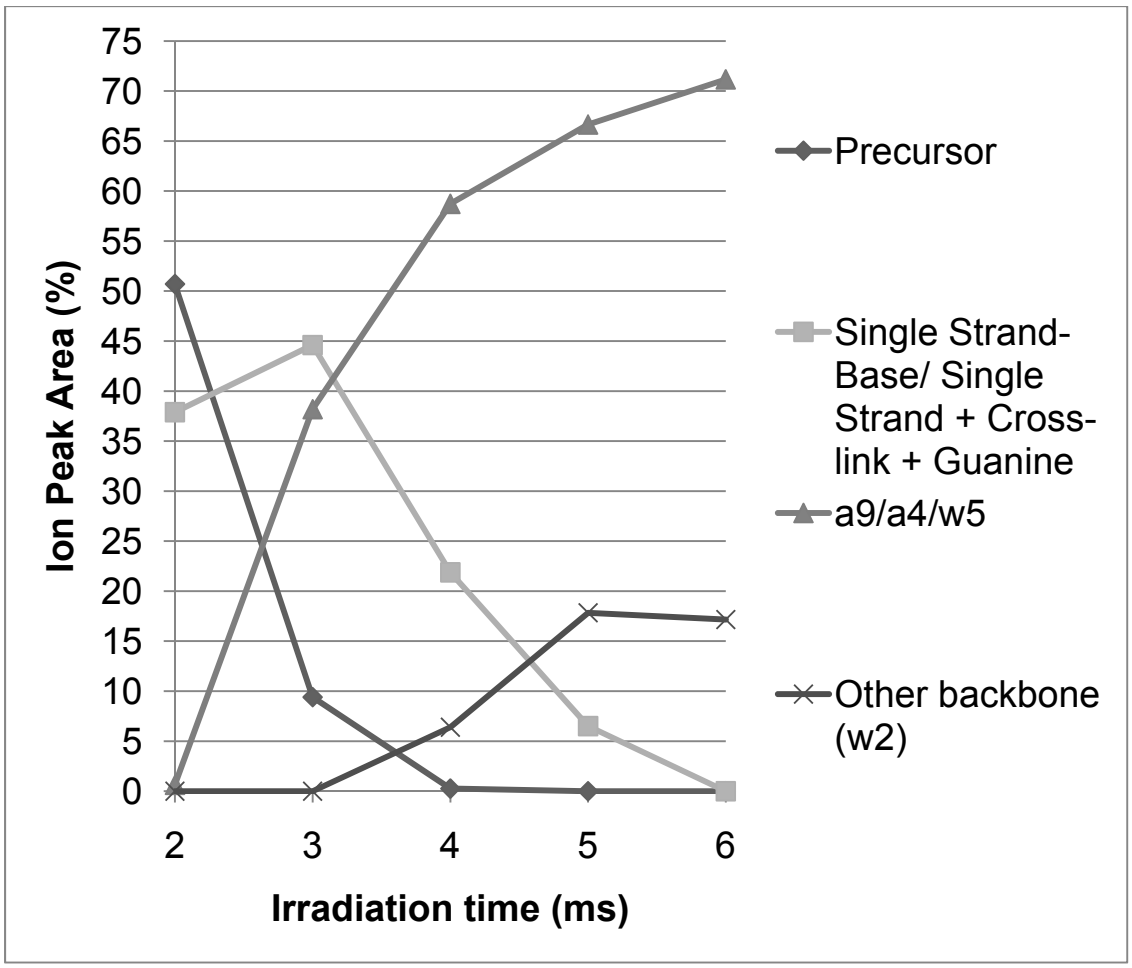


Figure 6.6 Time-variable IRMPD of $[DS4+RH1]^{6-}$

time is increased, the proportion of informative backbone fragments increases. Even longer irradiation times result in both an increase in backbone fragmentation at the site of cross-linking and additional backbone fragments remote from the cross-linking sites with the latter remaining relatively minor. The graph in Figure 6 indicates that the first dissociation event is cleavage between the cross-linked guanine and the sugar, leading to strand separation. These primary product ions then dissociate further to produce backbone fragments at the cross-linked nucleobase. These major backbone fragmentation patterns are summarized in Figure 7 for all five duplexes. Further irradiation leads to high energy fragments along the backbone away from the cross-linked guanines to form. Not only can IRMPD be used to identify cross-links and where they occur, but it can also be used to determine fragmentation pathways.

6.5 CONCLUSIONS

RH1 was found to form cross-links between oligonucleotide duplexes with several target binding sequences. In addition to two 5-dGNC sequences, RH1 formed cross-links at a binding site with a 5'-dGAAC sequence indicating that the cross-link is capable of extending an additional distance. However, a duplex containing a 5'-GC sequence did not have cross-links formed after the reaction suggesting that a minimum distance between the guanine bases is necessary for cross-links to form. A terminal binding site did not produce cross-links indicating that a local helical structure was necessary for cross-linking. The addition of the phenyl group did lower cross-linking efficacy with almost all of the duplexes studied. With one duplex, DS2, cross-linking

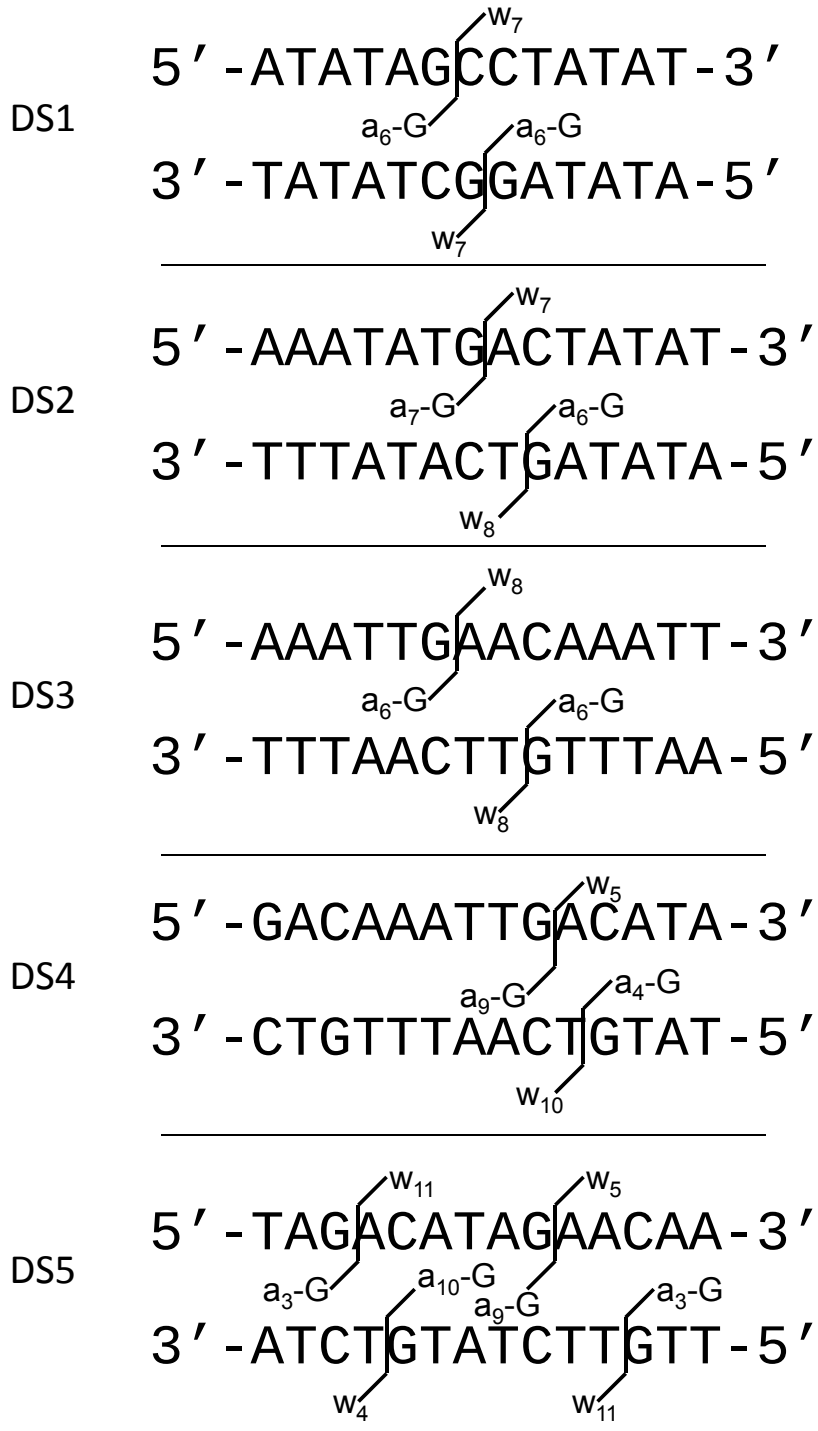


Figure 6.7 Major fragmentation pathways for duplexes cross-linked with RH1 or PhRH1

was similar between the two ligands suggesting that the binding site on this strand may have been more open than in the other duplexes. For the cross-linked duplexes, IRMPD provided useful information to determine where the cross-links occurred. The tunability of IRMPD, based on the irradiation times chosen, was used both to confirm the ligands were cross-linking and to determine how backbone fragments were forming. Further studies with various derivatives of RH1 will provide a greater understanding of the interaction between the aziridinylbenzoquinone cross-linkers and the target binding sequences.

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Chapter 7: Examination of the Effect of the Annealing Cation on Higher Order Structures Containing Guanine or Isoguanine Repeats

7.1 Overview

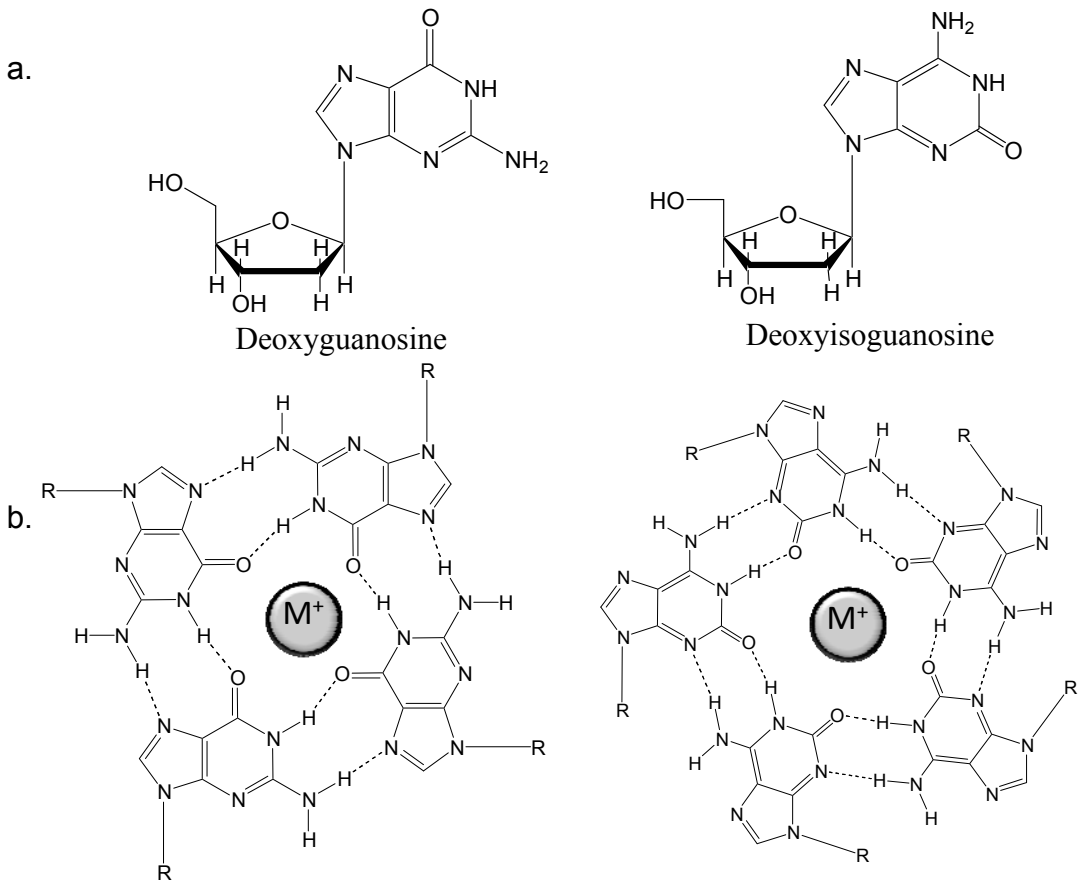
Isoguanine (2-oxo-6-amino-guanine), a natural but non-standard base, exhibits unique self-association properties compared to its isomer, guanine, and results in formation of different higher order DNA structures. In this work, the higher order structures formed by oligonucleotides containing guanine repeats or isoguanine repeats after annealing in solutions containing various cations are evaluated by electrospray ionization mass spectrometry (ESI-MS) and circular dichroism (CD) spectroscopy. The guanine-containing strand (G9) consistently formed quadruplexes upon annealing, whereas the isoguanine strand (Ig9) formed both pentaplexes and quadruplexes depending on the annealing cation. Quadruplex formation with G9 showed some dependence on the identity of the cation present during annealing with high relative quadruplex formation detected with six of ten cations. Analogous annealing experiments with Ig9 resulted in complex formation with all ten cations, and the majority of the resulting complexes were pentaplexes. CD results indicated most of the original complexes survived the desalting process necessary for ESI-MS analysis. In addition, several complexes, especially the pentaplexes, were found to be capable of cation exchange with ammonium ions. *Ab initio* calculations were conducted for isoguanine tetrads and pentads coordinated with all ten cations to predict the most energetically stable structures of the complexes in the gas phase. The observed preference of forming quadruplexes versus pentaplexes as a function of the coordinated cation can be

interpreted by the calculated reaction energies of both the tetrads and pentads in combination with the distortion energies of tetrads.

7.2 INTRODUCTION

The assembly of guanine base repeats in tetrads around central cations results in the formation of higher order DNA structures known as quadruplexes.[1] Quadruplexes have been studied extensively due to their role in halting both the immortalization of cells and tumor growth by preventing the binding of telomerase, a key enzyme responsible for the elongation of DNA.[2-5] Bases other than guanine are also capable of forming higher order DNA structures including triplexes, quadruplexes, and pentaplexes.[6] In addition to the growing body of evidence that supports the presence and function of higher order DNA structures formed by natural DNA bases, there is increasing interest in designing and exploiting the ability of both modified bases and non-standard bases to engage in intermolecular interactions, creating novel architectures for sensing applications and possible use as ion channels.[7-9]

Isoguanine, a non-standard but naturally occurring base, has been shown to form higher order complexes similar to guanine (Scheme 7.1).[8, 10-15] While the difference between guanine and isoguanine only involves the transposition of the carbonyl and amino groups, the change in the structure of isoguanine has been shown to favor the formation of pentameric rather than tetrameric multimers in studies of the interactions of the free bases with large cations.[16, 17] This striking change in multimeric assembly is



Scheme 7.1 a. Structures of deoxyguanosine and deoxyisoguanosine. b. Typical structures of guanine tetrad and isoguanine pentad around a central cation, M^+. R refers to the sugar or sugar phosphate backbone.

rationalized based on the decrease in the hydrogen bonding angle between bases which favors inclusion of a fifth base in the ring to maintain planarity. In addition, several computational studies have reported that isoguanine pentads formed around various monovalent cations have high interaction energies between bases and are planar complexes while the tetrads are far less planar.[18-21] Previous studies have noted a strong preference for the self-assembly of isoguanine around cesium over potassium ions.[22] In addition, individual isoguanosine units functioning as ionophores were found to be

selective for singly charged cations in the order $Cs^+ >> Rb^+ > K^+ > Na^+$ and for doubly charged cations in the order $Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$.[15] The cation in the central cavity also has a significant template role in the formation of multimeric DNA structures from guanine- and isoguanine-containing strands. Both monovalent and divalent cations can support the formation of quadruplexes from guanine-rich strands.[23-27] In contrast, isoguanine-containing strands form pentaplexes around cesium cations but form quadruplexes around potassium, whereas the analogous guanine-containing strands consistently form quadruplexes.[28] The impact of the central cation on the formation and stability of pentaplexes by isoguanine-containing strands has not been systematically explored.

ESI-MS has been increasingly employed as a versatile and sensitive tool for the study of higher order DNA complexes.[29, 30] While most of the studies have focused on quadruplexes[31-36] and their interactions with drugs or small molecules,[37-44] DNA triplexes have also been characterized.[45] ESI-MS allows ready assessment of the stoichiometries of complexes containing different numbers of cations and differentiation of single strand and multimeric species. Circular dichroism (CD) is also an established technique for studying biomolecular structures, including guanine quadruplexes in which the stacking of guanine bases affords strong optical activity.[42, 46-50] While CD is rather unresponsive to changes in fine structure or the sequences of the strands involved in the quadruplexes, it is sensitive to the overall conformations of the quadruplexes.[51] In addition, most salts and buffers are compatible within the range of wavelengths of interest for studying DNA complexes. Polyisoguanine strands have also been studied previously by CD

spectroscopy[52] as have complexes composed of individual bases around a central cation.[15]

Unlike guanine quadruplexes and other non-Watson-Crick interactions[53], isoguanine quadruplexes and pentaplexes have not been studied experimentally in detail. However, high level *ab initio* calculations provide an alternative approach to predict the structures of higher order DNA complexes. While the study of full quadruplex or pentaplex complexes is extremely resource-intensive due to the large number of atoms involved in the calculations, high level *ab initio* calculations have been carried out on smaller-scale isoguanine tetrads and pentads.[18-22] Most investigations completed previously focused on alkali metal ions alone and did not explore a full range of metals cations.

In this work, the formation of quadruplexes and/or pentaplexes by strands containing either guanine repeats or isoguanine repeats is studied by ESI-MS and CD spectroscopy. The strands are annealed in solutions containing a variety of singly charged and doubly charged template cations including Li^+, Na^+, K^+, Rb^+, Cs^+, NH_4^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+}. The wide range in ionic radii of these cations allows investigation of the effects of charge and ion size on the formation of higher order complexes. ESI-MS is used to determine the extent of complex formation as well as the identity and number of the central cations incorporated in the complexes. CD spectroscopy provides information about the structures and conformations of the complexes and is used to reveal how sample preparation affected the structures of interest. *Ab initio* optimizations with density functional theory are undertaken for

isoguanine tetrad and pentad structures incorporating all ten cations. For each complex, three different symmetries, specifically C_{4h}, D_4 and S_8 for isoguanine tetrads and C_{5h}, D_5 and S_{10} for isoguanine pentads, are investigated. The computational results are used to support the experimentally observed trends concerning the formation of quadruplexes and pentaplexes by isoguanine-containing strands.

7.3 EXPERIMENTAL

7.3.1 Materials.

The isoguanine-containing strand studied here, dTIgIgIgIgTTTT (Ig9), where Ig refers to isoguanine, was synthesized as described previously.[7] The corresponding guanine-containing strand, dTGGGGTTTT (G9), was obtained from IDT DNA, Inc (Coralville, IA). Spectroanalyzed methanol, HPLC grade water, and ammonium acetate were purchased from Thermo Fisher Scientific (Waltham, MA). Lithium chloride, rubidium chloride, cesium chloride, magnesium chloride dihydrate, calcium chloride dehydrate, strontium chloride hexahydrate, and barium chloride were purchased from Sigma Aldrich (St. Louis, MO). Sodium chloride and potassium chloride were obtained from EM Science (Gibbstown, NJ). All salts were dissolved in water to make stock solutions. Ionic radii are based on Goldschmidt hard sphere ionic radii.[54]

7.3.2 Methods.

Both G9 and Ig9 were annealed in a similar manner. The strands (100-125 μM single strand) and appropriate salts at concentrations described in the text were placed in

a hot water bath and cooled to room temperature overnight (approximately 12 hours). The annealed solutions were placed in the freezer until analyzed. Immediately before ESI-MS analysis, all solutions except those containing ammonium acetate were desalted using Millipore (Billerica, MA) Biomax Ultrafree 5 kDa molecular weight centrifugal filters. After desalting, ammonium acetate and methanol were added to the solutions to assist desalting and enhance desolvation of the analytes. The ammonium acetate (50 mM final concentration) and methanol (20% by volume) were added no more than two minutes before analysis. Assuming complete formation of the quadruplexes, in the case of G9, or pentaplexes, in the case of Ig9, the final concentrations of the complexes were approximately 3 μM. ESI-MS analysis was performed on a Thermo LTQ mass spectrometer (San Jose, CA). Analysis was performed in the negative mode with a heated capillary temperature of 90^{o}C and a spray voltage of 3.5 kV. The tube lens voltage was approximately -250 V.

To estimate the relative complex formation of quadruplexes or pentaplexes from the ESI mass spectra, peak areas of the complexes were summed and divided by the peak areas of the complexes and the free single strands as shown in the following equation:

$$\textit{Relative Complex Formation} = \frac{PA_Q \text{ or } PA_P}{PA_Q + PA_P + PA_{SS}} \tag{7.1}$$

where PA_Q and PA_P refer to the peak areas of the quadruplex and pentaplex species, respectively, and PA_{SS} refers to the peak area of the single strand. There were no pentaplexes detected for the G9 solutions, resulting in $PA_P = 0$ for all G9 solutions. Results discussed in this work are the average of three analyses.

Annealed solutions of G9 and Ig9 were also analyzed by CD spectroscopy via a Jasco (Easton, MD) J-815 circular dichroism instrument. The solutions were analyzed in a 0.1 cm cuvette over a wavelength range of 320 to 220 nm. Three sets of spectra were collected for each mixture: the first after simple dilution in water to 300 μl, the second after filtration with the 5kDa molecular weight filters as described above, and the third after adding ammonium acetate to the filtered solution so that the final concentration of ammonium acetate in the solution was 50 mM. Five scans were averaged for each spectrum. For the solutions analyzed before desalting, individual blanks containing the salt used for annealing were subtracted through the software. A water blank and a 50 mM ammonium acetate blank were subtracted from the spectra taken after desalting and the spectra taken after addition of ammonium acetate, respectively. CD spectra were not normalized against one another.

For each complex, three conformations representing three molecular point groups were constructed for *ab initio* optimizations using B3LYP/lacv3p**.[55] This basis set, which utilizes effective core potentials for K^+, Rb^+, Cs^+, Ca^{2+}, Sr^{2+}, and Ba^{2+}, and 6-311G** for the other cations, offers a reasonable balance between efficiency and accuracy in studying complexes containing metallic elements. The molecular symmetry was enforced during the optimization. All the calculations were performed using the Jaguar module (version 7.5) of Schrodinger, LLC. Considering that the formation of isoguanine quadruplexes requires the tetrad planes be parallel to each other, a constrained optimization that enforces the planarity of tetrad was employed for each of the tetrads. Technically, the cation was placed in the origin and the tetrad planes were perpendicular

to the Z axis. The constraint was imposed by making the Z-coordinates *z* for the four isoguanines above the XY plane and -*z* for the four isoguanines below the XY plane, where *z* is an optimized parameter. Similar conditions were used for the isoguanine pentad calculations. It should be noted that neither a frequency analysis nor BSSE (basis set superposition error) calculation was performed since the major purpose was to study the geometries of the complexes and to compare the relative template ability of the ten cations to promote the formation of isoguanine tetrads and pentads. According to recent literature, the zero point and BSSE corrections should not change the overall trends.[18, 20]

7.4 RESULTS AND DISCUSSION

7.4.1 ESI-MS analysis of G9.

G9 was annealed in the presence of ten different cations, and the resulting solutions were analyzed by ESI-MS after removal of excess salts. Representative spectra from these solutions are shown in Figure 7.1. The spectra obtained for G9 with Li^+, Mg^{2+}, Na^+, and Cs^+ show little or no presence of quadruplexes, either due to lack of formation during annealing or due to dissociation of the quadruplexes during clean-up (as discussed more later). In contrast, the spectra obtained for G9 with Sr^{2+}, K^+, NH_4^+, Ba^{2+}, Rb^+ displayed large abundances of quadruplexes. G9 annealed in Ca^{2+} resulted in approximately equal abundances of single strands and quadruplexes. The results are summarized in bar graph form in Figure 7.2 in the order of increasing ionic radius of the annealing cation. It is clear that the cations within a certain range of radii afford far more

efficient quadruplex formation as opposed to cations with very large (Cs^+) or small (Li^+) radii.

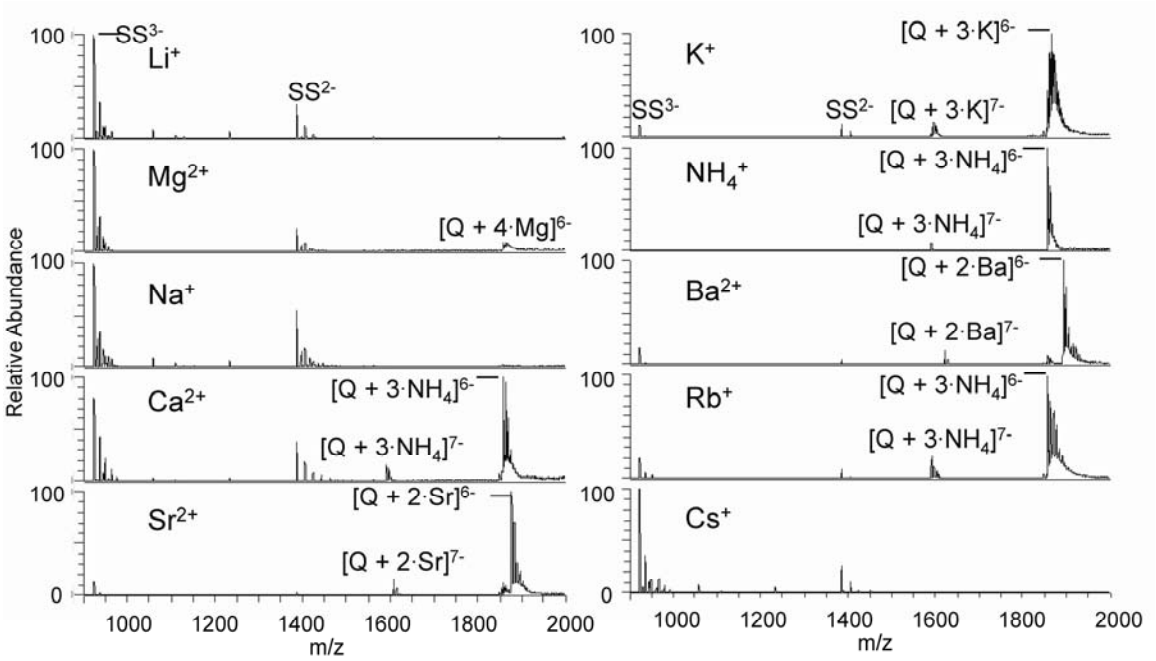


Figure 7.1 ESI-mass spectra of G9 after annealing in a 440mM solution of the salt noted in the spectra. Q refers to the quadruplex while ss denotes the single strand.

In addition to allowing the ready assessment of the efficiency of quadruplex formation, ESI-MS allows the determination of the identity and number of central cations present within the structures, as summarized for ions in the dominant 6- charge states in Table 7.1. For most of the solutions, the ESI mass spectra revealed that the number of central cations in the dominant form of the quadruplex was three, along with far less abundant complexes containing varying numbers of cations. This is consistent with a pattern reported previously in which n - 1 central cations are associated with n number of tetrads in a tetramolecular quadruplex.[56] Based on the G9 sequences used in the present

study, the formation of four tetrads is possible, supporting the inclusion of three cations. Overall, the number of cations included generally followed the n - 1 rule for singly

Table 7.1 Table comparing the identity and number of cations found associated with the 6- charge state of the G9 intermolecular quadruplex. Asterisks denote those quadruplexes in which the annealing cations are replaced by ammonium ion.

Most abundant Q^{6-} ion		
Cation	m/z	# cations
Li^+	1857	3*
Mg^{2+}	1864	4
Na^+	1861	3
Ca^{2+}	1857	3*
Sr^{2+}	1877	2
K^+	1867	3
NH_4^+	1857	3
Ba^{2+}	1894	2
Rb^+	1857	3*
Cs^+	1857	3*

charged cations. However, for the divalent cations Sr^{2+} and Ba^{2+}, only two cations were associated with the quadruplexes. While these cations are large, several monovalent cations that were larger or the same size promoted the incorporation of three cations. Thus, it appears that the greater charge density of Sr^{2+} and Ba^{2+} prevents the inclusion of a third cation in the quadruplex. In contrast to the results for the solutions containing Sr^{2+} and Ba^{2+}, four Mg^{2+} ions were incorporated in the quadruplexes. This ion, with a radius

approximately half that of Ba^{2+}, is the smallest one retained within the complex. It appears that quadruplex formation and cation retention are related to both the size and charge of the annealing cations.

In several cases, the m/z values of the quadruplexes in the mass spectra indicate that the central cations are not the original templating cations used during annealing but instead are ammonium ions. Quadruplexes in which the cations were replaced by ammonium are denoted by a box in Figure 7.2. Ammonium acetate is added to each solution after the desalting procedure that is essential for successful ESI-MS analysis of solutions containing high concentrations of non-volatile salts. Cation exchange within G-quadruplexes has been observed previously by Ma et al. and attributed to the sequential replacement of cations of lower affinity with those of higher affinity.[31] The quadruplexes that undergo exchange of the annealing cations for NH_4^+ include those originally annealed in the presence of Li^+, Ca^{2+}, Rb^+, and Cs^+. In fact, the Li^+ and Cs^+ solutions produced very low abundance quadruplexes, possibly indicative that these quadruplexes were only formed after the addition of ammonium acetate and never actually incorporated the very small Li^+ or very large Cs^+ ions (supported by CD results discussed below). However, both the Ca^{2+} and Rb^+ solutions exhibited high abundances of quadruplexes, indicating the ability to facilitate quadruplex formation despite the subsequent exchange with ammonium. Rb^+ is the only cation that was larger than ammonium in terms of ionic

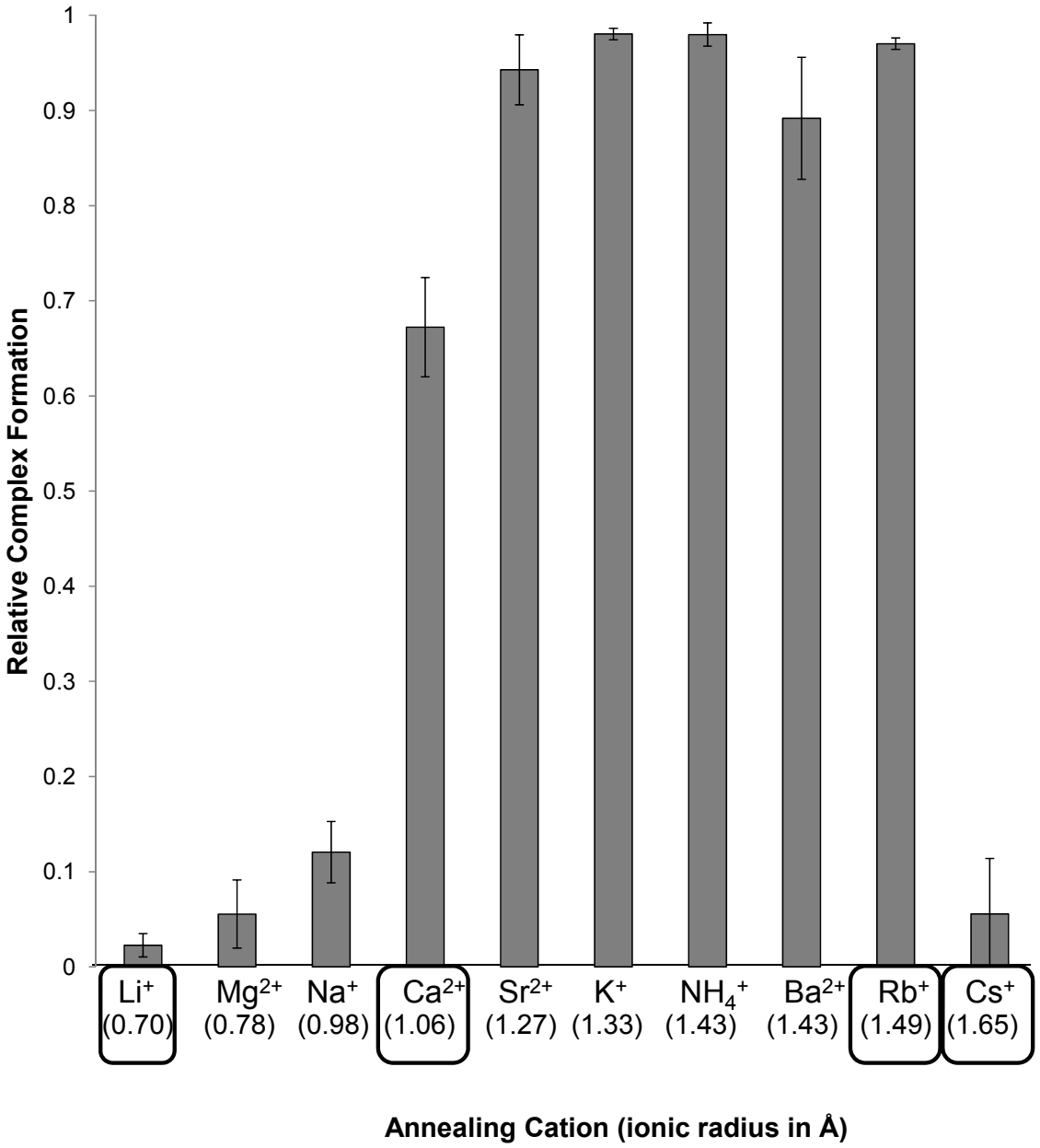


Figure 7.2 Chart of relative quadruplex formation for G9 annealed in 440 mM salt as determined from the ESI-mass spectra. Boxes around the cations indicate that the ESI-mass spectra showed replacement of the annealing cations with ammonium.

radius yet was still able to promote quadruplex formation. The larger size of Rb^+ and rather different electrostatic interactions may have contributed to its ready exchange for NH_4^+ prior to ESI-MS analysis. The formation of quadruplexes in the presence of Ca^{2+} and then subsequent exchange of Ca^{2+} for NH_4^+ is interesting as Ca^{2+} is the only divalent cation to exchange with NH_4^+. The other two doubly charged cations, Sr^{2+} and Ba^{2+}, for which quadruplexes were very abundant, were larger than Ca^{2+}. It is possible that the size and charge density of Ca^{2+} was suitable for facilitating the initial quadruplex assembly but ultimately the greater NH_4^+ affinity caused cation exchange during the desalting and ammonium acetate dilution required for ESI-MS analysis. In general, the exchange of template cations for ammonium seen here is consistent with previous results reported by Ma et al.[31]

7.4.2 ESI-MS of Ig9.

As with G9, the annealed Ig9 solutions were analyzed by ESI-MS after desalting. Representative spectra are shown in Figure 7.3. The Ig9 solutions showed large relative abundances of higher order complexes versus the single strands for all cations regardless of size or charge. In addition, the majority of the solutions resulted in almost entirely pentaplex formation with minimal quadruplex formation except for the Ig9 annealed in K^+ and NH_4^+ solutions. Even the smallest singly charged cation, Li^+, promoted almost exclusive pentaplex formation. Ig9 annealed in the NH_4^+ solution resulted in approximately equal abundances of quadruplexes and pentaplexes. As summarized in Figure 7.4, the change in the relative abundances of quadruplexes and pentaplexes

between solutions containing different annealing cations is striking, even for those cations with similar ionic radii. For example, Sr^{2+}, with an ionic radius of 1.27 Å, stabilizes the formation of the pentaplex, whereas K^+, with an ionic radius of 1.33 Å, preferentially stabilizes the quadruplex. In this case, the increased charge density of Sr^{2+} likely plays a role in pre-organizing the wider central cavity of the pentaplex as opposed to the quadruplex. A similar situation is seen for the NH_4^+ and Rb^+ solutions in which the ionic radii, 1.43 Å for NH_4^+ and 1.49 Å for Rb^+, are very similar, whereas the distributions of quadruplexes and pentaplexes present are quite different. Rb^+ promoted only pentaplex formation yet annealing in NH_4^+ yielded both quadruplexes and pentaplexes. There appears to be a narrow range of acceptable ion sizes capable of supporting the formation of quadruplexes for Ig9 that is only met by K^+ and, to a lesser extent, NH_4^+. In contrast, the pentaplexes are formed in the presence of cations of a broad range of sizes and charge densities.

The mass spectra of the Ig9 solutions were also examined to determine the number and identity of the included central cations. In most of the pentaplex complexes, the central cations appeared to have been replaced by ammonium cations. This was true for Li^+, Mg^{2+}, Na^+, Ca^{2+}, and Rb^+. Also, the pentaplexes contained three central cations which is consistent with the (n - 1) rule seen for guanine-based quadruplexes. Only the pentaplexes formed around the large doubly charged cations, $Sr^{2+}$and Ba^{2+}, retained these cations. The pentaplexes annealed in Cs^+ retained two cesium cations with one additional NH_4^+ cation. Given the apparent preference for three cations in the central cavity, it appears that one NH_4^+ cation replaced one Cs^+ cation during the de-salting and

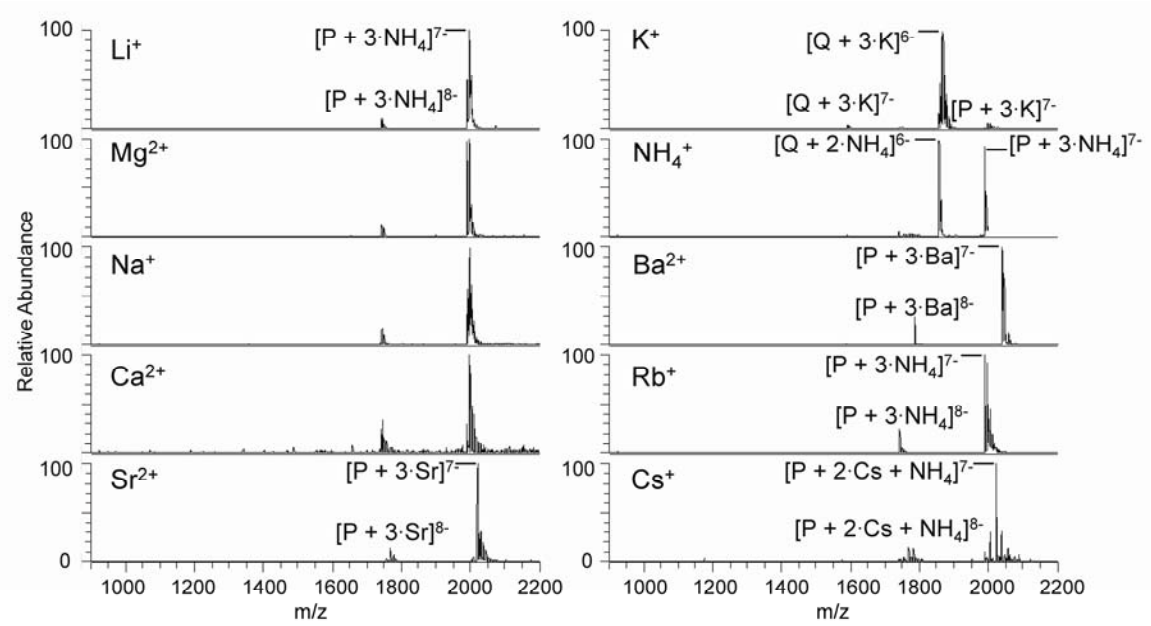


Figure 7.3 ESI-mass spectra of Ig9 after annealing in a 440 mM solution of the salt noted in the spectra. Q refers to the quadruplex and P refers to the pentaplex..

ammonium acetate dilution procedure. The Ig9 annealed in a K^+ solution resulted in both quadruplexes and low abundance pentaplexes that incorporated three K^+ cations.

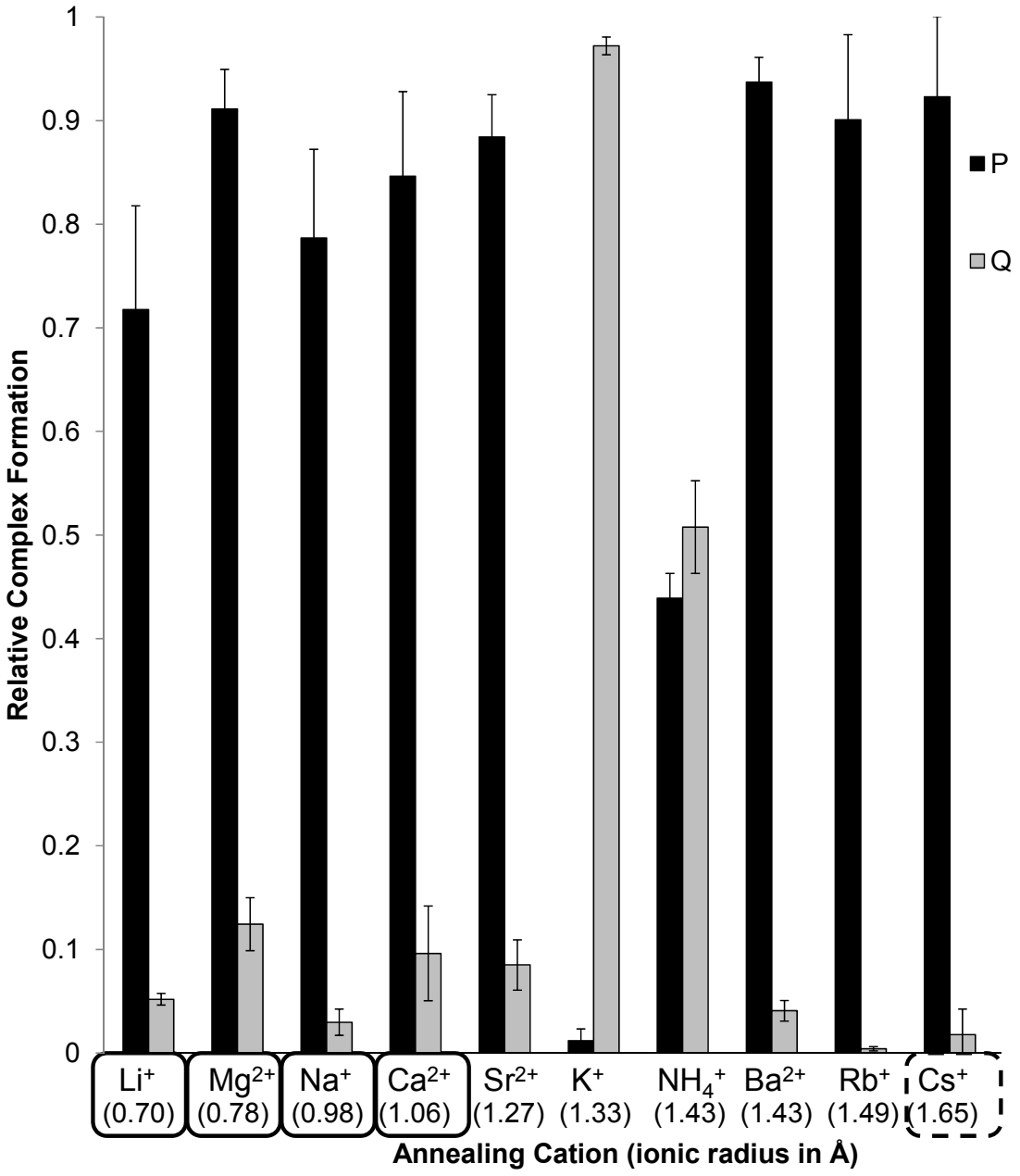


Figure 7.4 Chart of relative complex formation for Ig9 annealed in 440 mM salt as determined from the ESI-MS. The values represent the average of three samples. Boxes around the cations indicate that the ESI-mass spectra showed replacement of the annealing cations with ammonium. Only one of three Cs^+ cations was exchanged.

7.4.3 ESI-MS of G9 and Ig9 annealed in low salt concentration solutions.

To determine the effects of salt concentration during annealing on complex formation, both G9 and Ig9 were annealed in solutions containing 10 mM or 150 mM salts. After annealing, the resulting solutions were desalted in a similar fashion to solutions annealed in 440 mM salt, re-constituted in 50 mM ammonium acetate, and analyzed by ESI-MS. The results obtained for the G9 solutions are summarized in Figure 7.5a. In several cases, there was virtually no change in the relative abundances of quadruplexes when compared to the ESI mass spectra obtained for the high salt concentration solutions discussed in Figure 7.2. G9 annealed in Sr^{2+}, K^{+}, NH_4^{+}, and Ba^{2+} retained high levels of quadruplexes, while Li^{+}, Ca^{2+}, and Cs^{+} showed similar low abundances of quadruplexes. The two cases of greatest interest were the G9s annealed in Ca^{2+} and Rb^{+}. For these solutions, the decrease in salt concentration of the annealing solution resulted in a corresponding decrease in quadruplex formation, indicating that the stability of these quadruplexes is dependent on the salt concentration of the solution and suggesting that the quadruplexes are less robust. In addition, the spectra of solutions of G9 annealed in Mg^{2+} were also different than the spectra obtained at high annealing concentrations. The peaks corresponding to the quadruplexes incorporating Mg^{2+} were much broader than usually observed (spectra not shown), a feature that indicates the quadruplexes possess different numbers and identities of cations, thus yielding a range of complexes rather than a single dominant complex as was observed with the other cations.

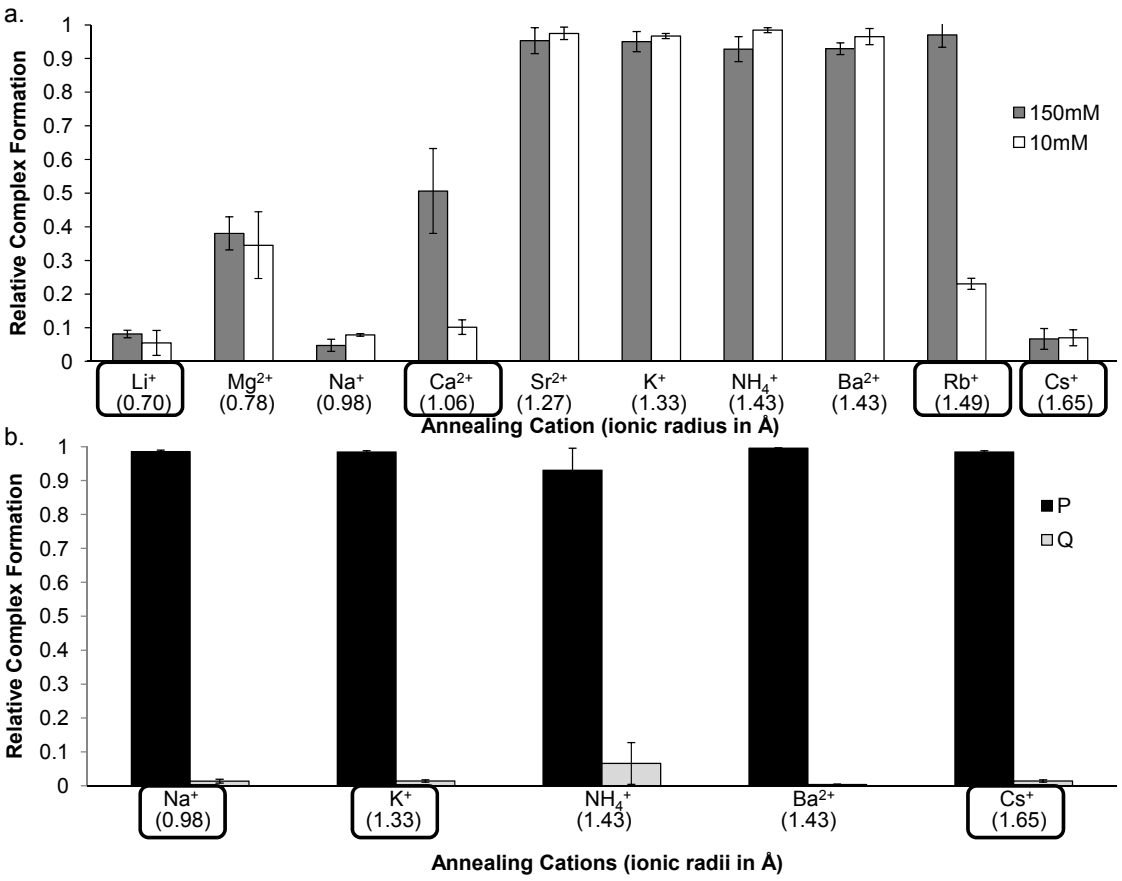


Figure7.5 Charts of relative complex formation under low ionic strength conditions for G9 quadruplexes (a) and Ig9 quadruplexes and pentaplexes (b). G9 was annealed in 150 mM and 10 mM salts and Ig9 was annealed in 10 mM salts. The values represent the average of three samples. Boxes around the cations indicate that the ESI mass spectra showed replacement of the annealing cations with ammonium.

CD results discussed below indicate that Mg^{2+} is capable of templating quadruplex formation with G9 although the structures do not survive desalting. While the overall quadruplex formation did not change significantly between 150 mM and 10 mM salt concentrations, these results do suggest a difference in stability.

The impact of the salt concentration on the formation of higher order complexes of Ig9 was evaluated more selectively due to the limited quantity of Ig9. Ig9 was

annealed in 10 mM Na^+, K^+, NH_4^+, Ba^{2+}, or Cs^+, a subset of cations that includes a range of ionic radii. The relative abundances of pentaplexes and quadruplexes under these conditions are summarized in Figure 7.5b. Ig9 annealed in the presence of 10 mM Na^+, Ba^{2+}, or Cs^+ resulted in similar distributions of complexes to those obtained for the solutions annealed at higher salt concentrations (seen in Figure 7.5). However, there were notable differences for the 10 mM K^+ and NH_4^+ solutions. Unlike Ig9 annealed at high salt conditions, the solutions annealed in 10 mM K^+ or NH_4^+ resulted in formation of mostly pentaplexes rather than quadruplexes that dominated at higher salt concentrations. In addition, the isoguanine pentaplexes formed around potassium at low salt concentrations tended to exhibit loss of an isoguanine base. It is unclear why the loss of isoguanine would occur solely under these conditions since it did not occur for other solutions annealed in the presence of 10 mM cations (i.e. low salt conditions) nor did the loss of isoguanine base occur for the solutions annealed in the presence of high concentrations of potassium. The differences seen for the solutions containing potassium and ammonium suggest that the pentaplexes are more stable than the quadruplexes under low salt conditions, but that the quadruplex can be stabilized at higher salt concentrations. Because the solutions containing G9 or Ig9 retained different distributions of complexes depending on the nature of the salt, it is apparent that the desalting and ammonium acetate dilution procedure used for processing all the solutions did not cause systematic disassembly and re-organization of the complexes to one uniform species.

7.4.4 CD spectra of G9 and Ig9.

To further evaluate the structures of the quadruplexes and pentaplexes formed by G9 and Ig9, respectively, and to determine the impact of the desalting and ammonium acetate dilution procedures used for the ESI-MS analysis, the solutions described above were analyzed by CD spectroscopy before desalting (after annealing in 440 mM salt), after desalting, and after addition of ammonium acetate (to 50 mM). Both the quadruplex structures and pentaplex structures formed by G9 and Ig9 are expected to provide good CD spectra as stacking between the bases, which is present in quadruplexes and pentaplexes, imparts optical activity to the structures. Since the salts are not significantly optically active under circularly polarized light from 220 to 320 nm, the presence of these salts does not hinder analysis of the solutions. Representative CD spectra are shown in Figures 7.6 and 7.7 for the G9 and Ig9 solutions, respectively, and all the results are summarized in Tables 7.2 and 7.3.

The quadruplexes formed by G9 are parallel tetramolecular quadruplexes which have a positive band around 264 nm and a smaller negative band around 243 nm. Table 7.2 provides a summary of peak locations and intensities for all solutions. Solutions of G9 annealed in the presence of Na+, Sr2+, K+, NH4+, Ba2+, and Rb+ produced strong positive bands at 264 nm before desalting. The band intensities at 264 nm were moderate for the Mg2+ and Ca2+ solutions, and low for the Li^{+} and Cs^{+} solutions. Examples of strong, moderate, and weak positive bands produced by solutions annealed in K^{+}, Mg^{2+}, and Cs^{+}, respectively, are shown in Figure 7.6a and Figure 7.6b before and after desalting.

Table 7.2 Wavelength maxima of positive peaks and intensities at 264 nm from CD spectra from solutions of G9.

	Peaks (nm)				CD intensity at 264 nm (mdeg)		
	Before Desalting	After Desalting	After NH_4OAc		Before Desalting	After Desalting	After NH_4OAc
Li^+	257	257	258	Li^+	0.61	0.29	0.47
Mg^{2+}	260	262	259	Mg^{2+}	2.09	0.33	0.59
Ca^{2+}	261	264	260	Ca^{2+}	2.66	0.60	0.80
Na^+	265	259	259	Na^+	4.54	0.39	0.54
Sr^{2+}	263	263	263	Sr^{2+}	3.49	2.21	1.88
Ba^{2+}	264	264	263	Ba^{2+}	4.01	2.01	1.98
K^+	264	264	264	K^+	4.88	3.22	2.97
NH_4^+	263	-	264	NH_4^+	3.92	-	3.49
Rb^+	264	264	264	Rb^+	4.69	2.64	1.90
Cs^+	256	256	260	Cs^+	0.45	0.18	0.36

When compared to the CD spectrum of a DNA solution at 95 ^oC, which is a sufficiently high temperature to convert all higher order structures to single strands, the CD spectra acquired for the solutions annealed in Li^+ and Cs^+ are similar in both spectral shape and intensity, indicating that Li^+ and Cs^+ do not favor higher order structure formation, which is in agreement with the ESI-MS results. The loss of base stacking as the quadruplex converts to the single strand diminishes the optical activity and decreases the corresponding CD intensities, indicating a loss of secondary structure. The majority of the CD spectral findings are consistent with the ESI-MS results for quadruplex formation discussed above in terms of the relative abundances of quadruplexes, with the exception of the results for Mg^{2+} and Na^+. Based on the mass spectral data, G9 annealed in the presence of Mg^{2+} and Na^+ displayed low abundances of quadruplexes whereas the prevalence of the quadruplexes is high in the CD spectra.

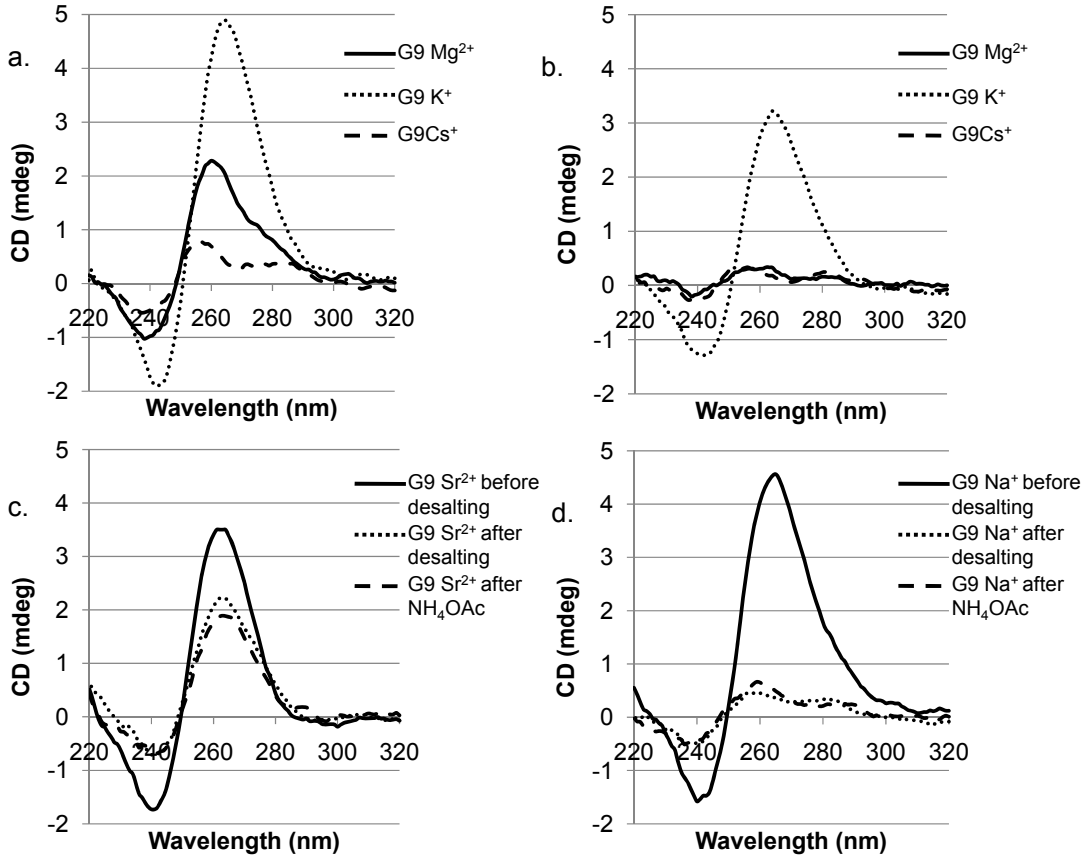


Figure 7.6 CD spectra of G9 samples annealed in the presence of Mg^{2+}, K^+, and Cs^+ before desalting (a) and after desalting (b). Initial salt concentrations, after dilution to working volumes, was approximately 18 mM. Assuming complete quadruplex formation, the concentration of quadruplex after dilution was approximately 3 μM. CD spectra before desalting, after desalting, and after the addition of ammonium acetate for samples annealed in the presence of Sr^{2+} (c) and Na^+ (d).

To investigate the stability and survival of the quadruplexes, as well as to probe the reasons for the differences in the relative abundances of Mg^{2+} and Na^+ complexes obtained from the ESI-MS results versus the CD results, CD spectra were collected for the same solutions after desalting and after dilution in ammonium acetate, thus reproducing the conditions used for the ESI-MS analysis (see 7.6c and d for example

spectra). Table 7.2 contains data for all samples. The resulting spectra show that quadruplexes are maintained, albeit at lower abundances, for G9 annealed in Sr^{2+}, K^{+}, Ba^{2+}, and Rb^{+}, even after desalting, and the spectral features remain consistent with parallel quadruplexes. However, the CD spectra for G9 annealed in the presence of Mg^{2+} and Na^{+} exhibit blue shifts for the bands from 264 nm to 257 nm and from 243 nm to 238 nm, as well as notable decreases in overall intensities which indicates a structural change, most likely denaturation to the single strands. Figure 7.6d highlights the changes seen for the quadruplexes annealed in the presence of Na^{+}. This CD data suggests that the quadruplexes formed around the Mg^{2+} and Na^{+} cations are less stable than those formed around other template cations as the salt concentration in solution decreases.

The final set of experiments entailed the examination of the CD spectra of the desalted G9 solution after the addition of ammonium acetate. Since quadruplexes can form around NH_4^{+} cations, as has been seen in both the ESI-MS data and the CD spectra, it was necessary to see if the addition of ammonium acetate after annealing and desalting would affect the survival of the original quadruplexes formed in the initial annealing step. For all of the solutions, the CD results indicate no dramatic change in the prevalence of the quadruplexes after addition of ammonium acetate (refer to CD results summarized in Table 7.2). The solutions in which quadruplexes showed low prevalence after desalting (Li^{+}, Mg^{2+}, Na^{+}, Ca^{2+}, and Cs^{+}) displayed only slight increases in intensities after the addition of ammonium acetate. The solutions in which the initial quadruplexes were more prevalent (Sr^{2+}, K^{+}, Ba^{2+}, and Rb^{+} solutions) showed modest declines in intensities. This series of results suggests that the surviving quadruplexes are predominantly ones

formed during the initial annealing procedure and that some of the less strongly bound template cations (Li^+, Ca^{2+}, Rb^+, Cs^+) can be exchanged for NH_4^+ without complete disruption or collapse of the quadruplex structures.

Five Ig9 solutions were chosen for further study by CD spectroscopy, and representative spectra are shown in Figure 7.7 for solutions annealed in Na^+, K^+, and Ba^{2+}. The CD spectra obtained for Ig9 annealed in the presence of Na^+, NH_4^+, Ba^{2+}, and Cs^+ are similar, whereas the spectra obtained for Ig9 in the presence of K^+ are quite different (Figure 7.7a). Overall, the intensities of the spectral features are lower than observed for the G9 solutions, possibly indicating a lower degree of planarity because the CD signals for these complexes largely arise from base stacking. The CD spectra of Ig9 annealed with Na^+, NH_4^+, Ba^{2+}, and Cs^+ exhibit strong positive bands at 307 nm and weaker negative bands around 289 nm. The wavelengths and intensities of spectral features are summarized in Table 7.3. These features are similar to the CD spectrum previously reported for poly(isoguanylic acid) with a positive band at 295 nm and a negative band around 275 nm. The prior measurements for poly(isoguanylic acid) were undertaken in 10 mM sodium chloride.[52] Similar results were also obtained for individual isoguanine bases bound to modified sugars when analyzed in dichloromethane with various cations present.[15]

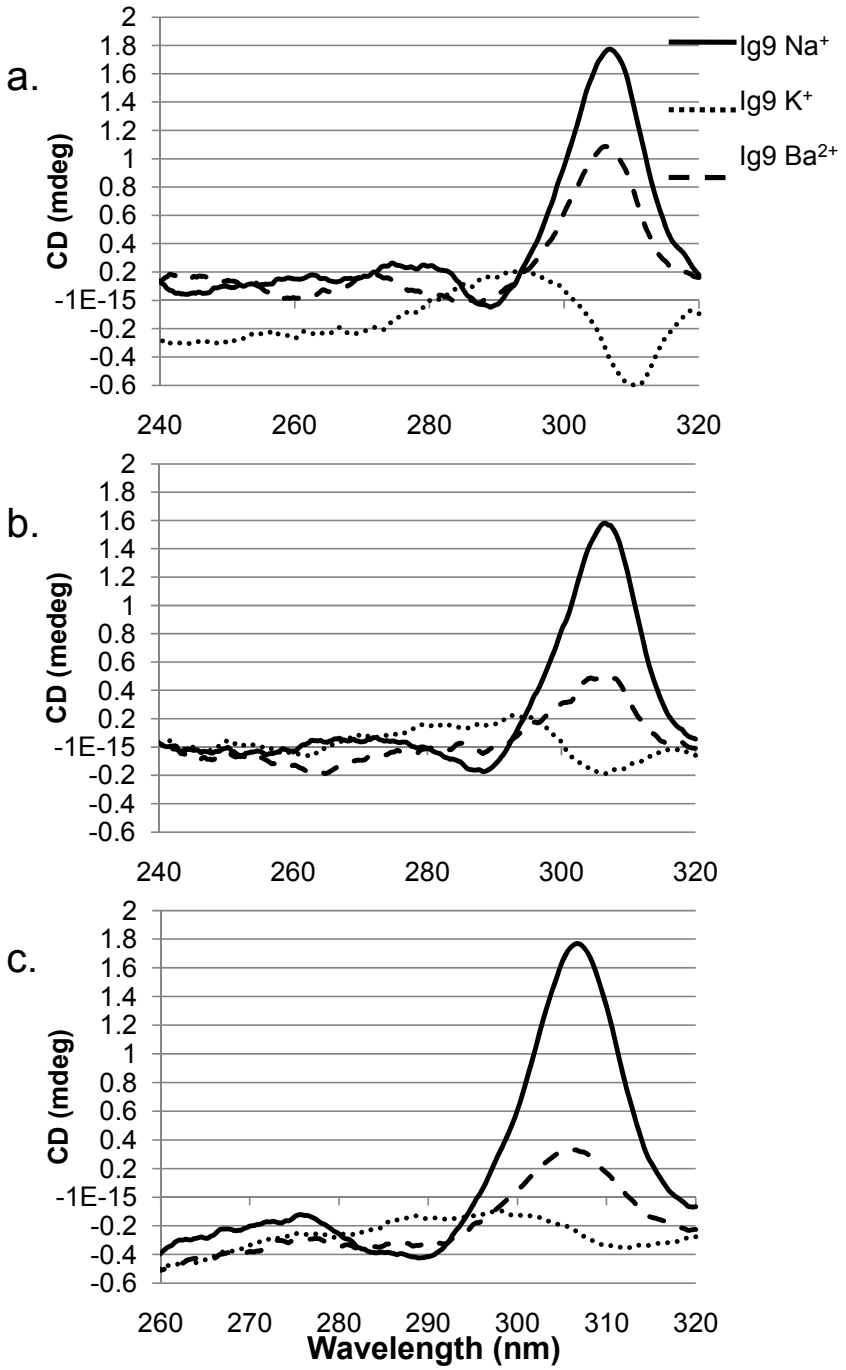


Figure 7.7 CD spectra of Ig9 samples annealed in the presence of Na^+, K^+, and Ba^{2+} before desalting (a), after desalting (b), and after the addition of ammonium acetate (c).

Table 7.3 Wavelength maxima and corresponding intensities of major CD spectral features from solutions of Ig9

Peak/Trough (nm)				CD intensity at peak/trough (mdeg)			
	Before Desalting	After Desalting	After NH_4OAc		Before Desalting	After Desalting	After NH_4OAc
Na^+	307	307	307	Na^+	1.77	1.58	1.77
$K^{+[a]}$	310	306	312	$K^{+[a]}$	-0.60	-0.19	-0.36
Cs^+	308	308	308	Cs^+	0.93	0.75	1.00
Ba^{2+}	306	306	306	Ba^{2+}	1.09	0.50	0.33
NH_4^+	307	306	306	NH_4^+	0.60	0.71	0.47

[a] K^+ results are reported for the trough since the peak for this sample was relatively less intense.

The CD spectrum obtained for Ig9 annealed in K^+ was very different from the spectra obtained for the solutions containing other cations with a strong negative band at 310 nm and a weak positive band at 293 nm (Figure 7.7a). These bands contrast significantly from from the CD features of G9 annealed in K^+, which had a positive band at 264 nm and a negative band at 241 nm (Figure 7.6a), thus highlighting the impact of the change in bases from guanine to isoguanine. Despite the differences between the spectra, ESI-MS results indicate that both the G9 and Ig9 strands form quadruplexes under these conditions. The notable differences in CD spectra between the quadruplexes presumably formed by Ig9 and G9 suggest significant conformational re-organization that accompanies the change from guanine to isoguanine in the strands. The isoguanine quartets are not anticipated to be as planar as the guanine quartets, a factor which would reduce the effects of base stacking interactions and lead to distorted stacked tetrads. Moreover, the change from guanine to isoguanine certainly alters the optical properties of

the resulting quadruplexes due to the different structures of the constituent bases and their interactions.

Upon desalting of the Ig9 solutions, there are slight decreases in the peak intensities (Figure 7.7b) that are restored after dilution of the solutions in ammonium acetate (Figure 7.7c). The solution annealed in Ba^{2+} showed the greatest relative decrease in intensity after desalting and the addition of ammonium acetate, which may point to greater instability of the Ba^{2+} pentaplex in low salt environments.

7.4.5 Ab initio calculations of isoguanine complexes.

To help rationalize some of the experimental findings regarding the formation of pentaplexes versus quadruplexes for the isoguanine strands in the presence of different cations, high-level *ab initio* optimizations were performed on three molecular symmetries for the smaller scale isoguanine complexes. As an example of the complexes formed, the optimized geometries of the Na^+ complexes are shown in Figures 7.8a and 7.8b for the tetrads and pentads, respectively, and the corresponding B3LYP/lacv3p** energies are listed in Table 7.4 for the tetrads and pentads. In each case S_8 is the most stable conformation for the tetrads containing most of the cations except for Rb^+, Cs^+ and NH_4^+, which have relatively larger ionic radii than the other cations. For the pentads, S_{10} is the most stable conformation for all five alkali metal complexes and the ones containing NH_4^+ and Ba^{2+}. In the optimized structures, the two $(isoguanine)_5$ pentad units are approximately parallel. Conversely, a significant degree of distortion was observed for

Table 7.4 The energies of the isoguanine tetrads and pentads in three symmetries at the B3LYP/lacv3p** level.

Tetrad	$E(S_8)$	$E(C_{4h})$ $-E(S_8)$	$E(D_4)$- $E(S_8)$	Pentad	$E(S_{10})$	$E(C_{5h})$ $-E(S_{10})$	$E(D_5)$- $E(S_{10})$
	Hartree	kcal/ mol	kcal/ mol		Hartree	kcal/ mol	kcal/ mol
Li^+	-4349.291856	8.83	0.28	Li^+	-5434.732948	15.09	8.46
Na^+	-4504.083846	6.80	0.93	Na^+	-5589.536896	16.07	9.51
K^+	-4369.934679	4.32	3.52	K^+	-5455.400848	8.95	3.29
Rb^+	-4365.651431	2.84	-0.98	Rb^+	-5451.139677	19.63	8.73
Cs^+	-4361.649461	-7.95	-0.59	Cs^+	-5447.157049	16.61	9.61
NH_4^+	-4398.873927	1.23	-5.65	NH_4^+	-5484.364241	11.58	10.23
Mg^{2+}	-4541.688798	17.90	1.04	Mg^{2+}	-5627.099895	61.98	-8.23
Ca^{2+}	-4378.232217	11.47	0.77	Ca^{2+}	-5463.642931	19.80	-1.15
Sr^{2+}	-4372.114214	8.26	0.45	Sr^{2+}	-5457.537268	6.36	-3.36
Ba^{2+}	-4366.895045	0.70	0.75	Ba^{2+}	-5452.363727	9.38	0.06

the analogous tetrads, and double-bowl-like conformations were observed. The distortion parameter, d1, which measures the depth of the "bowl" is listed in Table 7.5 for each tetrad. Generally, the structures incorporating larger cations exhibited greater distortions. As the pentads are relatively planar, the d1 symmetry is not calculated for these complexes. However, the distances between the two planar $(isoguanine)_5$ units tended to increase with the radius of the cation (Table 7.5). The distortion in the tetrad planes compared to the planarity of the pentads supports the dominance of pentaplexes over quadruplexes for strands with isoguanine repeats.

To measure the relative template ability of each cation to promote isoguanine tetrads and pentads, the reaction energies, ΔE, were calculated. ΔE and the equations to obtain ΔE are listed in Table 7.6 along with the energies of the complexes and ions

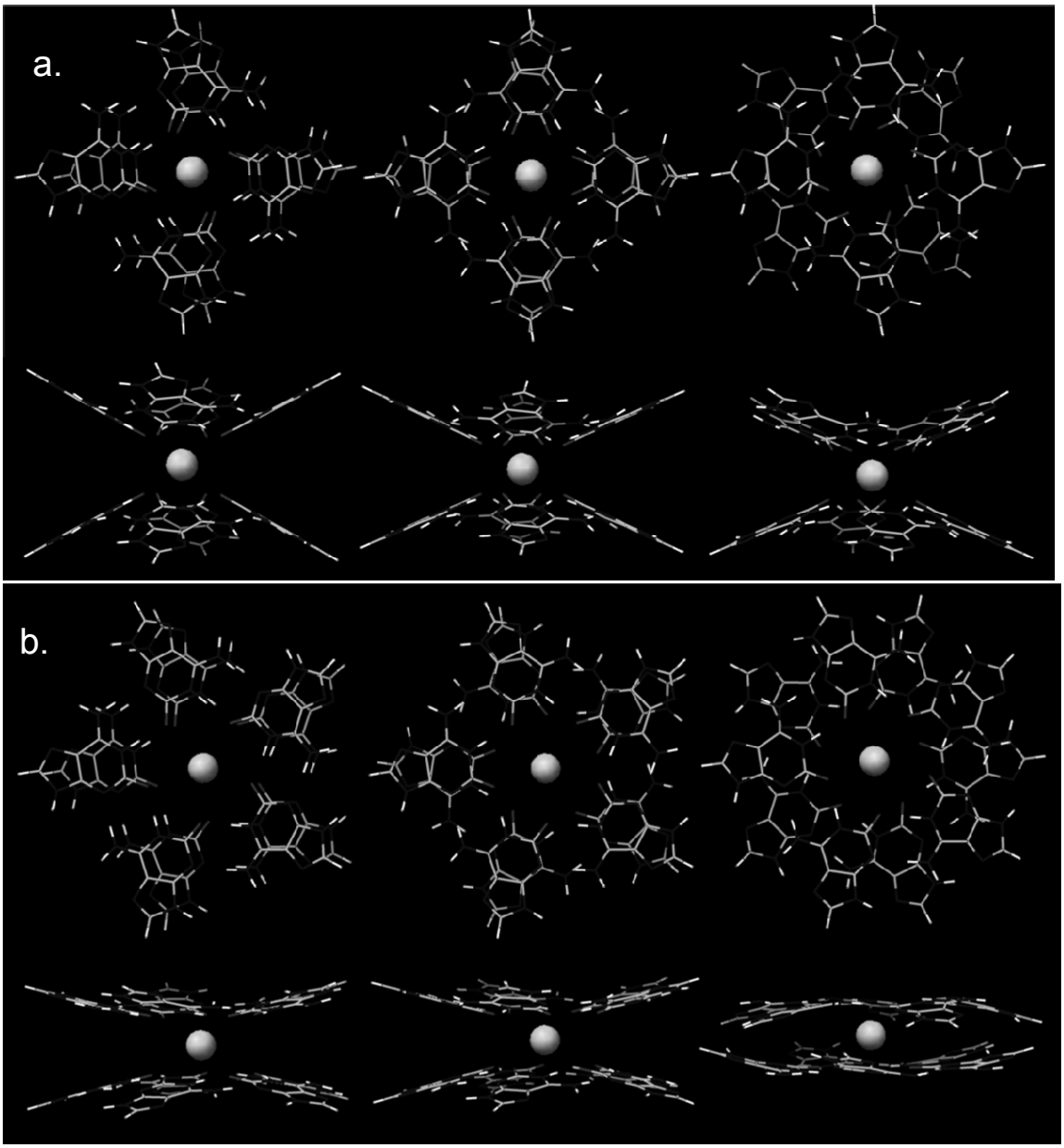


Figure 7.8 DFT optimized geometries of the isoguanine tetrads and pentads coordinated with Na^+. For the tetrads, three molecular symmetries, i.e. C_{4h}, D_4 and S_8, are shown from left to right (a). Top: the top views; bottom: the side views. For the pentads, C_{5h}, D_5 and S_{10}, are shown from left to right (b). Top: the top views; bottom: the side views.

Table 7.5 The distortion parameters of isoguanine tetrads and the distances between isoguanine pentad units. For the tetrads, *d1* is the distance between *Plane 1* and *Plane 2*. *d2* is the distance between the metal and the center of *Plane 2*. For the pentads, the metal is located in the geometric center of the pentad units.

Tetrad	Symmetry	d1 (Å)	d2 (Å)	Pentad	Symmetry	Distance (Å)
Li^+	S_8	2.366	1.254	Li^+	S_{10}	3.43
Na^+	S_8	2.363	1.283	Na^+	S_{10}	3.33
K^+	S_8	2.845	1.612	K^+	S_{10}	3.34
Rb^+	D_4	2.707	1.729	Rb^+	S_{10}	3.63
Cs^+	C_{4h}	3.32	2.138	Cs^+	S_{10}	3.73
NH_4^+	D_4	3.09	1.66	NH_4^+	S_{10}	4.08
Mg^{2+}	S_8	2.572	1.18	Mg^{2+}	D_5	3.07
Ca^{2+}	S_8	2.5	1.284	Ca^{2+}	D_5	2.91
Sr^{2+}	S_8	2.968	1.425	Sr^{2+}	D_5	2.87
Ba^{2+}	S_8	2.343	1.549	Ba^{2+}	S_{10}	2.93

individually. For the tetrads, the relative reaction energies, ΔΔE, increased moving down the periodic table. In contrast, this trend was not observed for the pentads as those incorporating K^+ have larger ΔΔE values than those containing either Na^+ or Rb^+. It should be noted that the trends changed after a hydration energy correction was performed. For example, after the hydration energy correction, the ΔΔE values determined for the tetrad complexes incorporating either Na^+, K^+ or NH_4^+ were similar, and generally much more favorable than the values for the complexes incorporating the other monovalent cations. On the other hand, ΔΔE values for pentads were more favorable with Rb^+, Cs^+ and NH_4^+ after the hydration energy correction. Overall, the energies of the pentads tended to be lower than the tetrads, supporting the tendency of the isoguanine strands to form pentaplexes.

Table 7.6 Table of energies of isoguanine tetrad and pentad formation using density functional theory at the B3LYP//lacv3p** level. *ΔΔE* is the relative reaction energy. For the alkali metal and ammonium complexes, the reference is the Li^+ complex, while the reference is the Mg^{2+} complex for earth alkali metal complexes.

Tetrad	Symmetry	$E_{Complex}$ hartree	E_{ion} hartree	ΔE hartree[a]	ΔΔE kcal/mol
Li^+	S_8	-4349.291856	-7.284906	-0.484702	0.00
Na^+	S_8	-4504.083846	-162.08746	-0.474138	6.63
K^+	S_8	-4369.934679	-27.970936	-0.441495	27.11
Rb^+	D_4	-4365.652992	-23.705936	-0.424808	37.58
Cs^+	C_{4h}	-4361.66213	-19.732039	-0.407843	48.23
NH_4^+	D_4	-4398.882934	-56.920092	-0.440594	27.68
Mg^{2+}	S_8	-4541.688798	-199.240881	-0.925669	0.00
Ca^{2+}	S_8	-4378.232217	-35.883928	-0.826041	62.52
Sr^{2+}	S_8	-4372.114214	-29.812995	-0.778971	92.05
Ba^{2+}	S_8	-4366.895045	-24.654361	-0.718436	130.04
Pentad	Symmetry	$E_{complex}$ hartree	E_{ion} hartree	ΔE hartree[b]	ΔΔE kcal/mol
Li^+	S_{10}	-5434.732948	-7.284906	-0.545232	0.00
Na^+	S_{10}	-5589.536896	-162.08746	-0.546626	-0.88
K^+	S_{10}	-5455.400848	-27.970936	-0.527102	11.38
Rb^+	S_{10}	-5451.139677	-23.705936	-0.530931	8.97
Cs^+	S_{10}	-5447.157049	-19.732039	-0.5222	14.45
NH_4^+	S_{10}	-5484.364241	-56.920092	-0.541339	2.44
Mg^{2+}	D_5	-5627.113004	-199.240881	-0.969313	0.00
Ca^{2+}	D_5	-5463.644766	-35.883928	-0.858028	69.83
Sr^{2+}	D_5	-5457.542618	-29.812995	-0.826813	89.42
Ba^{2+}	S_{10}	-5452.363727	-24.654361	-0.806556	102.13

[a] For the tetrads, ΔE, the reaction energy, is calculated using the following equation: $\Delta E = E_{complex} - (E_{ion} + 8\times E_{isoguanine})$, where the energy of isoguanine is - 542.690281 hartrees.

[b] For the pentads, ΔE, the reaction energy, is calculated using the following equation: $\Delta E = E_{complex} - (E_{ion} + 10\times E_{isoguanine})$ where the energy of isoguanine is - 542.690281 hartrees.

The distortion energy, which is the difference between the energies of the fully optimized structures and the partially optimized geometries with the enforcement of the planarity of the isoguanine units, is listed in Table 7.7 for each tetrad. Interestingly, tetrads containing NH_4^+ or K^+ had the smallest distortion energies of all the complexes. This implies that a smaller energetic penalty was paid by incorporation of NH_4^+ or K^+ upon formation of planar isoguanine tetrads compared to incorporation of other cations. This finding is consistent with the ESI-MS results showing that quadruplexes were the dominant species formed for strands annealed in high concentrations of K^+ and NH_4^+.

Table 7.7 The distortion energies of the most stable symmetry of isoguanine tetrads. The distortion energy, $E_{distortion} = E_{constrain} - E$, where $E_{constrain}$ is the energy of the partially optimized geometry forcing the two tetrad planes parallel to each other, and E is the energy of the fully optimized geometry. The distance between two isoguanine planes are also listed.

Tetrad	Symmetry	$E_{distortion}$ [kcal mol^{-1}]	Distance (Å)
Li^+	S_8	20.65	3.24
Na^+	S_8	16.48	3.33
K^+	S_8	13.73	3.52
Rb^+	D_4	17.43	3.79
Cs^+	C_{4h}	25.47	4.34
NH_4^+	D_4	11.66	3.78
Mg^{2+}	S_8	46.87	3.07
Ca^{2+}	S_8	38.52	3.21
Sr^{2+}	S_8	34.31	3.28
Ba^{2+}	S_8	29.55	3.42

The greater $\Delta\Delta E$ of the K^+-containing pentads in comparison to those incorporating neighboring cations Na^+ and Rb^+ may be another driving force for the formation of

quadruplexes over pentaplexes in the presence of K^+. The low distortion energies of the tetrads containing K^+ or NH_4^+ support the formation of isoguanine quadruplexes around these cations despite the lower energies of tetrads formed around smaller cations. The increased template abilities of K^+ and NH_4^+ with respect to formation of planar tetrad units appears to facilitate quadruplex formation over pentaplex formation under certain conditions despite the higher energy of the tetrads.

7.5 CONCLUSIONS

Quadruplexes and pentaplexes of G9 and Ig9, respectively, were annealed in solutions containing cations of varying charge and ionic radius. The formation of G9 quadruplexes was far more dependent on the identity of the central cation than the formation of Ig9 complexes. While Ig9 complexes were formed around all of the cations, annealing in only six of the ten cations produced high levels of G9 quadruplexes. In addition, five of various annealing cations, including Li^+, Mg^{2+}, Na^+, Ca^{2+}, and Rb^+, were not retained but instead were replaced by NH_4^+ in the Ig9 pentaplexes when ammonium acetate was added to the solutions immediately before ESI-MS analysis. This was only true for G9 quadruplexes annealed in the presence of Li^+, Ca^{2+}, Rb^+, and Cs^+. The ready exchange of central cations points to the utility of the Ig9 pentaplexes as efficient monovalent cation channels. Also, *ab initio* calculations indicate that the formation of planar isoguanine tetrads around the template cations K^+ and NH_4^+ require the least amount of energy, thus offering some rationalization for the formation of quadruplexes in the presence of high concentrations of these cations. CD results also confirm that many

of the complexes retain their higher order structure throughout the desalting process, thus indicating the complexes are resistant to degradation in low salt environments.

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Chapter 8: Conclusions

The work presented in this dissertation provides greater insight into the interactions of ligands with DNA and intermolecular interactions of DNA and the extent to which mass spectrometry can be used to further improve our understanding of those interactions. Binding affinities and selectivities were determined for non-covalent binding ligands while the binding affinities and binding sites were determined for covalently bound ligands. Covalent and non-covalent interactions are crucial for the both cellular function and for ligand binding, and insight into both types of interactions is valuable for the design of more selective DNA interactive agents or modulation of binding affinities.

The studies involving the non-covalent interactions of ligands to quadruplexes were described in chapters 3 and 4. The study of pyrrole inosine ligands, discussed in chapter 3, reported the use of ESI-MS to characterize non-covalent DNA interactions. Comparison of the binding of the three ligands indicated that the ligand designed to form a three-point Hoosteen interaction with guanine bound specifically to quadruplexes and guanine-rich duplexes. The ligand that could only form a two point interaction bound oligonucleotides with a variety of secondary structures regardless of the presence of guanines. When the third binding functionality was replaced by a bulky blocking group, binding to all sequences was greatly diminished. These results were obtained quickly and highlight the ability of ESI-MS to be used for determining the relative specificity of a

series of ligands. In addition, dissociation spectra were used to determine the type of non-covalent interaction observed with the ligands. The dissociation pattern indicated that the pyrrole inosine ligand stacked on the end of the quadruplexes and intercalated into the duplexes. These results were obtained during the same experiments used to determine the binding specificity providing valuable information without using additional time or sample. The Pt ligands that bound to quadruplex DNA were described in chapter 4. In this work, the size and shape of the Pt-centered complexes improved the interaction with quadruplexes in comparison to other ligands. A small Pt-based complex and a larger, non-planar Ru-based complex did not exhibit the same enhanced binding affinity to quadruplex oligonucleotides. In addition, it was determined that the specific structural motifs at the ends of the quadruplex, where the ligands bound, had an effect on whether the ligands would complex with the DNA. Binding to anti-parallel intramolecular quadruplexes or to tetramolecular quadruplexes was observed, but not binding to parallel intramolecular quadruplexes. These results, obtained through a combination of CD and MS analysis, highlights the advantages of mass spectrometry and how additional information gained through other complementary experiments can be used to more fully characterize DNA/ligand complexes.

In chapter 5 the interactions of anthracycline ligands, doxorubicin and daunorubicin, with both wild-type and mismatched hairpin DNA were evaluated. The ligands both exhibited enhanced binding to the mismatched sequences. Non-specific binding was observed when the concentration of ligand exceeded that of the DNA, resulting in complexes with high ligand:DNA stoichiometries. Mass spectrometry

allowed determination of the stoichiometries of the DNA/ligand complexes as well as afforded rapid screening of solutions with varying DNA/ligand concentration ratios.

The interstrand crosslinking of duplex DNA was discussed in chapter 6. Diaziridinyl benzoquinone crosslinkers were reduced *in vitro* and reacted with duplex DNA. Analysis by LC-MS indicated that crosslinks were formed with both crosslinkers when a 5'-GNC or 5'-GNNC sequence was present in the duplex. However, reactions with duplexes with 5'-GC or 5'-CG sequences did not result in crosslinks. In order for the crosslinks to form, it was necessary to have a minimum distance between opposite strand guanine bases. In addition, terminal target sequences did not result in the formation of crosslinks indicating that a specific secondary structure was necessary for crosslink formation. The diaziridinyl benzoquinone with a phenyl substituent did not form crosslinks as well as the methyl substituted benzoquinone indicating the bulky phenyl group limited the binding affinity or reactivity. ESI-MS was used not only to verify the formation of crosslinked products, but were also used to determine the site of crosslinking based on tandem mass spectrometry.

The final chapter explored non-covalent interactions between DNA strands. Strands containing either isoguanine or guanine were annealed with different monovalent or divalent cations to determine how those scaffold cations would affect the secondary structure of the DNA. While guanine-containing strands only formed quadruplexes and only formed them around a few selected cations, sequences incorporating isoguanine formed pentaplexes around most of the cations studied. Quadruplexes were observed even when the annealing cation was potassium or ammonium, but the structures formed

around the other cations, no matter the charge or cationic radius, were pentaplexes. Not only was the presence of four or five strands easily determined based on the m/z ratio of the complexes, both the identity and the number of cations present in the central cavity of the complex were also readily determined.

The work described in this dissertation demonstrates the range of information that can be gained from the mass spectrometric investigation of DNA complexes. In addition to determining the affinities and selectivities of ligands, binding sites can also be interrogated and determined by tandem mass spectrometry. These techniques can be used to study non-covalent and covalent DNA binding ligands especially for the identification and characterization of anti-cancer and anti-biotic agents which frequently bind to DNA. Given the speed and low sample sizes required for MS analysis, these techniques could be readily applied to high-throughput drug screening.

Future work in this area would certainly focus on using LC-MS to investigate covalent interactions and crosslinks. While the research presented in chapter 6 is the place to begin, work to improve detection limits would permit the investigation of even smaller samples and detection of crosslinks in vivo. This might be accomplished through even more sensitive separation/mass analyzer combinations or through improvements in the reaction efficiencies. In addition, using tandem mass spectrometry to determine relative crosslinking efficiencies of various binding/crosslinking sites would more easily permit the identification of ideal covalent binding sites. As this work is extended to larger ligands and, in the future, to peptides and proteins, determining a way to analyze

large DNA/protein complexes will be necessary as these large complexes are not easily detected by current mass spectrometric methods.

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